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Docket No. 54002-D/JPW/JHB

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

HON. COMMISSIONER OF PATENTS AND TRADEMARKS
Washington, D.C. 20231

December 15, 1998

S I R:

Transmitted herewith for filing are the specification and claims of patent application of:

Kenneth A. Jones, Thomas M. Laz, and Beth Borowsky for Inventor(s)

DNA Encoding A GABA_{R2} Polypeptide And Uses Thereof
Title of Invention

Also enclosed are:

☒ 51 sheet(s) of ☐ informal ☒ formal drawings.

☐ Oath or declaration of Applicant(s).

☐ A power of attorney

☐ An assignment of the invention to

☒ A Preliminary Amendment

☒ A verified statement to establish small entity status under 37 C.F.R. §1.9 and §1.27.

The filing fee is calculated as follows:

CLAIMS AS FILED, LESS ANY CLAIMS CANCELLED BY AMENDMENT

	NUMBER FILED		NUMBER EXTRA*		RATE		FEE		
					SMALL ENTITY	OTHER ENTITY		SMALL ENTITY	OTHER ENTITY
Total Claims	19 -20	=	0	X	\$ 9.00	\$ 18.00	=	\$ -0-	\$
Independent Claims	3 -3	=	0	X	\$ 39.00	\$ 78.00	=	\$ -0-	\$
Multiple Dependent Claims Presented: ___ Yes <u> X </u> No					\$ 130.00	\$ 260.00	=	\$ -0-	\$
*If the different in Col. 1 is less than zero, enter "0" in Col. 2					BASIC FEE			\$ 380.00	\$760.00
					TOTAL FEE			\$ 380.00	\$

Jones, et al.
U.S. Serial No.: Not Yet Known
Filed: Herewith
Letter of Transmittal
Page 2

☒ A check in the amount of \$380.00 to cover the filing fee.

☐ Please charge Deposit Account No. _____ in the amount of \$_____.

☒ The Commissioner is hereby authorized to charge any additional fees which may be required in connection with the following or credit any over-payment to Account No. 03-3125:

☒ Filing fees under 37 C.F.R. §1.16.

☒ Patent application processing fees under 37 C.F.R. §1.17.

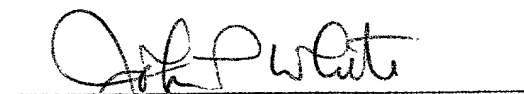
☐ The issue fee set in 37 C.F.R. §1.18 at or before mailing of the Notice of Allowance, pursuant to 37 C.F.R. §1.311(b).

☒ Three copies of this sheet are enclosed.

☒ A certified copy of previously filed foreign application No. _____ filed in _____ on _____.
Applicant(s) hereby claim priority based upon this aforementioned foreign application under 35 U.S.C. §119.

☒ Other (identify) one set of loose drawings; computer diskette containing
sequence listing; Statement in Accordance with 37 C.F.R.
§1.821(f); Express Mail Certificate of Mailing bearing label
No. EM 525 891 063 US dated December 15, 1998

Respectfully submitted,



John P. White
Registration No. 28,678
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Applicant or Patentee: Kenneth A. Jones, et al. Attorney's
 Serial or Patent No.: Not Yet Known Pocket No.: 54002-D
 Filed or Issued: Herewith
 Title of Invention or Patent: DNA Encoding A GABA_A R2 Polypeptide And Uses Thereof

VERIFIED STATEMENT (DECLARATION) CLAIMING
 SMALL ENTITY STATUS UNDER 37 C.F.R. §1.9(f)
 AND §1.37(c) - SMALL BUSINESS CONCERN

I hereby declare that I am:

- the owner of the small business concern identified below.
 X an official of the small business concern empowered to act on behalf of the
 concern identified below:

Name of Concern: Synaptic Pharmaceutical Corporation

Address of Concern: 215 College Road, Paramus, New Jersey 07652

I hereby declare that the above identified small business concern qualifies as a small business concern as defined in 13 C.F.R. §121.3-18, reproduced in 37 C.F.R. §1.9(d), for purposes of paying reduced fees under 35 U.S.C. §41(a) and §41(b), in that the number of employees of the concern, including those of its affiliates, does not exceed five hundred (500) persons. For purposes of this verified statement, the number of employees of the business concern is the average number, over the previous fiscal year, of the persons employed by the business concern on a full-time, part-time, or temporary basis during each pay period of the fiscal year, and concerns are affiliates of each other when, either directly or indirectly, one concern controls or has power to control the other, or a third party or parties controls or has power to control both.

I hereby declare that rights under contract or law have been conveyed to and remain with the small business concern identified above with regard to the invention entitled

DNA Encoding A GABA_A R2 Polypeptide And Uses Thereof
 described in:

 X the specification filed herewith
 application serial no. filed
 patent no. issued

If the rights held by the above identified small business concern are not exclusive, each individual, concern or organization having rights to the invention is listed below and no rights to the invention are held by any person, other than the inventor, who could not qualify as an independent inventor under 37 C.F.R. §1.9(c)*, any concern which could not qualify as a small business concern under 37 C.F.R. §1.9(d)* or as a nonprofit organization under 37 C.F.R. §1.9(e)*.

Name: N/A
 Address:
 Individual Small Business Concern Nonprofit Organization

*NOTE: Separate verified statements are required for each named person, concern, or organization having rights to the invention availing to their status as small entities. 37 C.F.R. §1.37.

*See Reverse

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37 C.F.R. §§1.9(d), 1.9(d), 1.9(e)

(c) An independent inventor as used in this chapter means any inventor who (1) has not assigned, granted, conveyed, or licensed, and (2) is under no obligation under contract or law to assign, grant, convey, or license, any rights in the invention to any person who could not likewise be classified as an independent inventor if that person had made the invention, or to any concern which would not qualify as a small business concern or a nonprofit organization under this section.

(d) A small business concern as used in this chapter means any business concern as defined by the Small Business Administration in 13 C.F.R. §§1.3-18, published on September 30, 1982 at 47 FR 43373. For the convenience of the users of these regulations, that definition states:

§121.3-18 Definition of small business for paying reduced patent fees under Title 35, U.S. Code.

(a) Pursuant to Pub. L. 97-347, a small business concern for purposes of paying reduced fees under 35 U.S. Code 41(a) and (b) to the Patent and Trademark Office means any business concern (1) whose number of employees, including those of its affiliates, does not exceed 500 persons and (2) which has not assigned, granted, conveyed, or licensed, and is under no obligation under contract or law to assign, grant, convey or license, any rights in the invention to any person who could not be classified as an independent inventor if that person had made the invention, or to any concern which would not qualify as a small business concern or a nonprofit organization under this section. For the purpose of this section concerns are affiliates of each other when either, directly or indirectly, one concern controls or has the power to control the other, or a third party or parties controls or has the power to control both. The number of employees of the business concern is the average over the fiscal year of the persons employed during each of the pay periods of the fiscal year. Employees are those persons employed on a full-time, part-time or temporary basis during the previous fiscal year of the concern.

(b) If the Patent and Trademark Office determines that a concern is not eligible as a small business concern within this section, the concern shall have a right to appeal that determination to the Small Business Administration. The Patent and Trademark Office shall transmit its written decision and the pertinent size determination file to the SBA in the event of such adverse determination and size appeal. Such appeals by concerns should be submitted to the SBA at 1441 L Street, NW., Washington, D.C. 20416 (Attention: SBA Office of General Counsel). The appeal should state the basis upon which it is claimed that the Patent and Trademark Office initial size determination on the concern was in error; and the facts and arguments supporting the concern's claimed status as a small business concern under this section.

(e) A nonprofit organization as used in this chapter means (1) a university or other institution of higher education located in any country; (2) an organization of the type described in section 501(c)(3) of the Internal Revenue Code of 1954 (26 U.S.C. 501(c)(3)) and exempt from taxation under section 501(a) of the Internal Revenue Code (26 U.S.C. 501(a)); (3) any nonprofit scientific or educational organization qualified under a nonprofit organization statute of a state of this country (35 U.S.C. 201(i)); or (4) any nonprofit organization located in a foreign country which would qualify as a nonprofit organization under paragraphs (e)(2) or (3) of this section if it were located in this country.

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Small Entity/Small Business Concern
Page -2-

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. 37 C.F.R. §1.26(b)*.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: Kathleen P. Mullins
Title in Organization: President
Address: Synaptic Pharmaceutical Corporation
215 College Road, Paramus, New Jersey 07652
Signature: Kathleen P. Mullins
Date of Signature: December 14, 1998

*See Reverse

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37 C.F.R. §1.34(b)

(b) Once status as a small entity has been established in an application or patent, fees as a small entity may thereafter be paid in that application or patent without regard to a change in status until the issue fee is due or any maintenance fee is due. Notification of any change in status resulting in loss of entitlement to small entity status must be filed in the application or patent prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate pursuant to §1.9 of this part. The notification of change in status may be signed by the applicant, any person authorized to sign on behalf of the assignee, or an attorney or agent of record or acting in a representative capacity pursuant to §1.34(a) of this part.

091455 124500

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Kenneth A. Jones, et al.
U.S. Serial No. : Not Yet Known
Filed : Herewith
For : DNA ENCODING A GABA_BR2 POLYPEPTIDE AND
USES THEREOF

1185 Ave of the Americas
New York, New York 10036
December 15, 1998

Assistant Commissioner for Patents
Washington, D.C. 20231

Sir:

PRELIMINARY AMENDMENT

Please amend the subject application as follows:

In the claims:

Please cancel claims 18-49, 51-81, and 83-189 without prejudice to applicants' right to pursue the subject matter of these claims in a future continuation or divisional application.

REMARKS

The Specification has been amended to update the continuing data for the subject application.

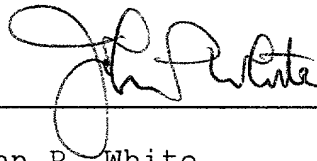
Claims 1-189 were pending in the subject application. By this Amendment applicants have canceled claims 18-49, 51-81, and 83-189. Accordingly, claims 1-17, 50, and 82 are currently pending.

If a telephone interview would be of assistance in advancing prosecution of the subject application, applicants' undersigned attorney invites the Examiner to telephone the number provided.

Kenneth A. Jones et al.
Serial No.: Not Yet Known
Filed: December 15, 1998
page 2

No fee, other than the enclosed fee of \$ 380.00 for filing this application, is deemed necessary in connection with the filing of this Preliminary Amendment. However, if any additional fee is required, authorization is hereby given to charge the amount of such fee to Deposit Account No. 03-3125.

Respectfully Submitted,

A handwritten signature in cursive script, appearing to read "John P. White", is written over a horizontal line.

John P. White
Registration No. 28,678
Attorney for Applicants
Cooper & Dunham LLP
1185 Avenue of the Americas
New York, New York 10036
(212) 278-0400

09211756 121698

**Application
for
United States Letters Patent**

To all whom it may concern:

Be it known that we,

Kenneth A. Jones, Thomas M. Laz, and Beth Borowsky

have invented certain new and useful improvements in

DNA ENCODING A GABA_BR2 POLYPEPTIDE AND USES THEREOF

of which the following is a full, clear and exact description.

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069421 5571.260

DNA ENCODING A GABA_B2 POLYPEPTIDE AND USES THEREOF5 BACKGROUND OF THE INVENTION

10 This application is a continuation-in-part of U.S. Serial No. 09/ , filed November 4, 1998 which is a continuation-in-part of PCT International Application No. PCT/US98/22033, filed October 16, 1998 which is a continuation-in-part of U.S. Serial No. 09/141,760, filed August 27, 1998, which is a continuation-in-part of U.S. Serial No. 08/953,277, filed October 17, 1997, the contents of which are hereby incorporated by reference into the subject application.

15 Throughout this application, various references are referred to within parentheses. Disclosures of these publications in their entireties are hereby
20 incorporated by reference into this application to more fully describe the state of the art to which this invention pertains. Full bibliographic citation for these references may be found at the end of this application, preceding the sequence listing and the
25 claims.

30 Gamma amino butyric acid (GABA) is the major inhibitory neurotransmitter in the nervous system. Three families of receptors for this neurotransmitter, GABA_A, GABA_B, and GABA_C, have been defined pharmacologically and genetically. GABA_B receptors were initially discriminated by their sensitivity to the drug baclofen (Bowery, 1993). This and their dependency on G-proteins for effector coupling distinguishes them from
35 the ion channel-forming GABA_A and GABA_C receptors. Principle molecular targets of GABA_B receptor activation are Ca⁺⁺ and K⁺ channels whose gating is directly modulated by the liberation of G-protein that follows the binding of the neurotransmitter to its receptor
40 (Misgeld et al. 1995; Krapivinsky et al., 1995a). In

this sense, GABA_B receptors operate mechanistically as other G-protein coupled receptors (GPCRs), such as dopamine D2, serotonin 5HT1a, neuropeptide Y and opiate receptors, that are also negatively coupled to adenylyl cyclase activity (North, 1989). Stimulation of GABA_B receptors inhibits release of neurotransmitters such as glutamate, GABA, somatostatin, and acetylcholine by modulation of Ca⁺⁺ and K⁺ channels at presynaptic nerve terminals. Inhibition of neurotransmitter release is one of the most prominent physiological actions of the GABA_B receptor and has provided a basis for the discrimination of receptor subtypes (Bowery et al. 1990). GABA_B receptors also mediate a powerful postsynaptic hyperpolarization of neuronal cell bodies via the opening of G-protein-gated inwardly rectifying K⁺ channels (GIRK) (Kofuji et al. 1996).

GABA_B receptors are widely distributed throughout the central nervous system. Receptor autoradiography and binding studies show that receptors are found in relatively high abundance in nearly all areas of the brain including cerebral cortex, hippocampus, cerebellum, basal ganglia, thalamus, and spinal cord (Bowery et al. 1987). In the periphery, GABA and GABA_B receptors are found in pancreatic islets, autonomic ganglia, guinea-pig ileum, lung, oviduct, and urinary bladder (Giotti et al. 1983; Erdo et al. 1984; Santicioli et al. 1986; Sawynok, 1986; Hills et al. 1989; Chapman et al. 1993).

Baclofen, the agonist that originally defined the GABA_B receptor subtype, has been used as an anti-spastic agent for the past 25 years. There is evidence in human that baclofen has a spinal site of action that most likely involves the depression of mono- and polysynaptic reflexes. In laboratory animals, baclofen has antinociceptive properties that are attributed to

the inhibition of release of excitatory neurotransmitters glutamate and substance P from primary sensory afferent terminals (Dirig and Yaksh, 1978; Sawynok, 1987; Malcangio et al., 1991). The presence of GABA_B receptors in intestine, lung and urinary bladder indicates a possible therapeutic role for diseases associated with these peripheral tissues. In spinal patients, baclofen is currently used for treatment of bladder-urethral dissynergia (Leyson et al., 1980). Selective GABA_B receptor agonists may also prove useful for the treatment of incontinence by reducing the feeling of bladder fullness (Taylor and Bates, 1979). Evidence from studies of the upper respiratory systems of cats and guinea-pigs suggests that GABA_B agonists also may be useful as antitussive agents and for the treatment of asthma (Luzzi et al., 1987; Bolser et al., 1993). In addition, GABA_B receptors have been implicated in absence seizure activity in the neocortex and with presynaptic depression of excitatory transmission in the spinal cord.

Studies of GABA_B receptor pharmacology and physiology have been greatly facilitated by the relatively recent arrival of potent and selective GABA_B receptor antagonists that are able to penetrate the blood-brain barrier. The most fruitful avenue for providing glimpses of GABA_B receptor subtypes has come from studies of neurotransmitter release. GABA, acting through GABA_B receptors, can inhibit the release of GABA, glutamate, and somatostatin in rat cerebrocortical synaptosomes depolarized with KCl. Three receptor subtypes have been hypothesized based on the potency of the agonists baclofen and 3-aminopropylphosphinic acid (3-APPA), and on the antagonists phaclofen and CGP35348 (Bonanno, Raiteri, 1992). For example, somatostatin release is inhibited

by baclofen and this effect is antagonized by phaclofen and CGP35348. Glutamate release is similarly affected except that the potency of phaclofen to block inhibition is considerably lower than that for release of somatostatin. A third receptor subtype, the cortical GABA autoreceptor, has been defined based on an insensitivity to CGP35348, although this potency difference is not seen in a cortical slice preparation (Waldmeier et al. 1994). In the spinal cord, the GABA autoreceptor is insensitive to baclofen, but sensitive to 3APPA and block by CGP35348. Interestingly, in this tissue baclofen is active at the GABA_B receptor modulating glutamate release. Differences in the sensitivities of presynaptic receptors controlling release of GABA and glutamate in the spinal cord may importantly contribute to the therapeutic action of baclofen as an antispastic agent (Bonanno, Raiteri, 1993).

Recently a polypeptide was isolated, GABA_BR1a, that binds radiolabelled GABA_B receptor antagonists in transfected cells (Kaupmann et al. 1997a). The predicted amino acid sequence displays homology with the metabotropic glutamate receptor gene family which includes eight members and a Ca⁺⁺-sensing receptor. Included in this homology is a large N-terminal domain that contains two lobes with structural similarity to the amino acid binding sites of bacterial proteins. A second polypeptide, GABA_BR1b, presumably a splice variant, differs from GABA_BR1a in that the N-terminal 147 amino acids are replaced by 18 different residues in the predicted mature protein after signal peptide cleavage. Transcripts for both GABA_BR1s are abundant and widely distributed in the rat brain. There appear to be differences in the localization of the splice variants in discrete regions of the brain, suggesting that their expression is differentially regulated

(Bischoff et al. 1997).

The pharmacological profile of the cloned GABA_BR1 polypeptide is similar in some respects to that of native receptors isolated from rat cerebral cortex, but there are important differences. For the high affinity antagonists studied, IC₅₀s are nearly identical to those at native receptors. In contrast, IC₅₀s for agonists and some low affinity antagonists display large rightward shifts relative to their displacement curves in native tissue. Additionally, both splice variants of the polypeptide couple poorly to intracellular effectors such as inhibition of adenylyl cyclase and, against expectations, fail completely to stimulate GIRK currents in oocytes (Kaupmann et al. 1997b). The poor binding affinity of agonists and weak or non-existent activation of effectors may not be adequately explained by inappropriate G-protein coupling in the heterologous expression system used.

The isolation by homology cloning of a novel polypeptide, GABA_BR2, from a human hippocampus cDNA library, as well the isolation of the rat homolog of the human polypeptide, is now reported. Also reported herein are functional assays involving the co-expression of the GABA_BR2 gene with a GABA_BR1 gene. These functional assays were not previously observed with the GABA_BR1 gene product alone. The pharmacological and signal transduction properties of the two gene products when expressed together match those of native GABA_B receptors in the brain. These functional assays permits high throughput screening for novel compounds having agonist or antagonist activity at the native GABA_B receptor.

SUMMARY OF THE INVENTION

This invention is directed to an isolated nucleic acid encoding a GABA_BR2 polypeptide.

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This invention is further directed to a purified GABA_BR2 protein.

10 This invention is further directed to a vector comprising the above-identified nucleic acid.

This invention is further directed to a above-identified vector, wherein the vector is a plasmid.

15 This invention is directed to a method of detecting a nucleic acid encoding a GABA_BR2 polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding
20 the GABA_BR2 polypeptide, wherein the probe has a unique sequence, which sequence is present within one of the two strands of the nucleic acid encoding the GABA_BR2 polypeptide contained in plasmid BO-55, and detecting hybridization of the probe to the nucleic acid.

25

This invention is further directed to a method of detecting a nucleic acid encoding a GABA_BR2 polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides,
30 which probe specifically hybridizes with the nucleic acid encoding the GABA_BR2 polypeptide, wherein the probe has a unique sequence, which sequence is present within (a) the nucleic acid sequence shown in Figures 22A-22D (Seq. ID No. 46) or (b) the reverse complement to the
35 nucleic acid sequence shown in Figures 22A-22D (Seq. ID No. 46), and detecting hybridization of the probe to the nucleic acid.

This invention is further directed to a method of detecting a nucleic acid encoding a GABA_BR2 polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the GABA_BR2 polypeptide, wherein the probe has a unique sequence, which sequence is present within one of the two strands of the nucleic acid encoding the GABA_BR2 polypeptide contained in plasmid pEXJT3T7-hGABAB2, and detecting hybridization of the probe to the nucleic acid.

This invention is further directed to a method of detecting a nucleic acid encoding a GABA_BR2 polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the GABA_BR2 polypeptide, wherein the probe has a unique sequence, which sequence is present within (a) the nucleic acid sequence shown in Figures 3A-3D (Seq. ID No. 3) or (b) the reverse complement to the nucleic acid sequence shown in Figures 3A-3D (Seq. ID No. 3), and detecting hybridization of the probe to the nucleic acid.

This invention is further directed to a method of detecting a nucleic acid encoding a GABA_BR2 polypeptide, which comprises contacting the nucleic acid with a probe comprising a nucleic acid of at least 15 nucleotides which is complementary to the antisense sequence of a unique segment of the sequence of the nucleic acid encoding the GABA_BR2 polypeptide, and detecting hybridization of the probe to the nucleic acid.

This invention is directed to an isolated antibody capable of binding to a GABA_BR2 polypeptide encoded by

the above-identified nucleic acid.

5 This invention is further directed to an antibody capable of competitively inhibiting the binding of the above-identified antibody to a GABA_BR2 polypeptide.

10 This invention is further directed to a pharmaceutical composition which comprises an amount of the above-identified antibody effective to block binding of a ligand to the GABA_BR2 polypeptide and a pharmaceutically acceptable carrier.

15 This invention is directed to a transgenic, nonhuman mammal expressing DNA encoding a GABA_BR2 polypeptide.

This invention is further directed to a transgenic, nonhuman mammal comprising a homologous recombination knockout of the native GABA_BR2 polypeptide.

20 This invention is further directed to a transgenic, nonhuman mammal whose genome comprises antisense DNA complementary to DNA encoding an above-identified GABA_BR2 polypeptide so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding such GABA_BR2 polypeptide and which hybridizes to such
25 mRNA encoding such GABA_BR2 polypeptide, thereby reducing its translation.

30 This invention is directed to a method of detecting the presence of a GABA_BR2 polypeptide on the surface of a cell which comprises contacting the cell with the above-identified antibody under conditions permitting binding of the antibody to the polypeptide, detecting the presence of the antibody bound to the cell, and
35 thereby detecting the presence of a GABA_BR2 polypeptide on the surface of the cell.

This invention is further directed to a method of preparing the purified GABA_βR2 polypeptide which comprises:

- 5 a. inducing cells to express a GABA_βR2 polypeptide;
- b. recovering the polypeptide so expressed from the induced cells; and
- 10 c. purifying the polypeptide so recovered.

This invention is further directed to a method of preparing the purified GABA_βR2 polypeptide which comprises:

- 15 a. inserting a nucleic acid encoding the GABA_βR2 polypeptide into a suitable vector;
- 20 b. introducing the resulting vector in a suitable host cell;
- c. placing the resulting cell in suitable condition permitting the production of the
- 25 GABA_βR2 polypeptide;
- d. recovering the polypeptide produced by the resulting cell; and
- 30 e. isolating or purifying the polypeptide so recovered.

This invention is directed to a GABA_βR1/R2 receptor comprising two polypeptides, one of which is a GABA_βR2 polypeptide and another of which is a GABA_βR1 polypeptide.

This invention is directed to a method of forming a GABA_BR1/R2 receptor which comprises inducing cells to express both a GABA_BR1 polypeptide and a GABA_BR2 polypeptide.

5

This invention is directed to an antibody capable of binding to a GABA_BR1/R2 receptor, wherein the GABA_BR2 polypeptide is encoded by the above-identified nucleic acid.

10

This invention is further directed to an antibody capable of competitively inhibiting the binding of the above-identified antibody to a GABA_BR1/R2 receptor.

15

This invention is directed to a pharmaceutical composition which comprises an amount of the above-identified antibody effective to block binding of a ligand to the GABA_BR1/R2 receptor and a pharmaceutically acceptable carrier.

20

This invention is directed to a transgenic, nonhuman mammal expressing a GABA_BR1/R2 receptor, which is not naturally expressed by the mammal.

25

This invention is further directed to a transgenic, nonhuman mammal comprising a homologous recombination knockout of the native GABA_BR1/R2 receptor.

30

This invention is directed to a method of detecting the presence of a GABA_BR1/R2 receptor on the surface of a cell which comprises contacting the cell with the above-identified antibody under conditions permitting binding of the antibody to the receptor, detecting the presence of the antibody bound to the cell, and thereby detecting the presence of a GABA_BR1/R2 receptor on the surface of the cell.

35

This invention is directed to a method of determining the physiological effects of varying levels of activity of GABA_BR1/R2 receptors which comprises producing an above-identified transgenic nonhuman mammal whose levels of GABA_BR1/R2 receptor activity vary due to the presence of an inducible promoter which regulates GABA_BR1/R2 receptor expression.

This invention is directed to a cell which expresses on its surface a mammalian GABA_BR1/R2 receptor that is not naturally expressed on the surface of such cell.

This invention is directed to a process for identifying a chemical compound which specifically binds to a GABA_BR1/R2 receptor which comprises contacting cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the GABA_BR1/R2 receptor.

This invention is directed to a process for identifying a chemical compound which specifically binds to a GABA_BR1/R2 receptor which comprises contacting a membrane fraction from a cell extract of cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the GABA_BR1/R2 receptor.

This invention is directed to a process involving competitive binding for identifying a chemical compound which specifically binds to a GABA_BR1/R2 receptor which comprises separately contacting cells expressing on

their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the GABA_BR1/R2 receptor, a decrease in the binding of the second chemical compound to the GABA_BR1/R2 receptor in the presence of the chemical compound indicating that the chemical compound binds to the GABA_BR1/R2 receptor.

This invention is directed to a process involving competitive binding for identifying a chemical compound which specifically binds to a human GABA_BR1/R2 receptor which comprises separately contacting a membrane fraction from a cell extract of cells expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the GABA_BR1/R2 receptor, a decrease in the binding of the second chemical compound to the GABA_BR1/R2 receptor in the presence of the chemical compound indicating that the chemical compound binds to the GABA_BR1/R2 receptor.

This invention is directed to a method of screening a plurality of chemical compounds not known to bind to a GABA_BR1/R2 receptor to identify a compound which specifically binds to the GABA_BR1/R2 receptor, which comprises

- (a) contacting cells containing nucleic acid encoding and expressing on their cell surface

the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with a compound known to bind specifically to the GABA_BR1/R2 receptor;

5

(b) contacting the same cells as in step (a) with the plurality of compounds not known to bind specifically to the GABA_BR1/R2 receptor, under conditions permitting binding of compounds known to bind the GABA_BR1/R2 receptor;

10

(c) determining whether the binding of the compound known to bind specifically to the GABA_BR1/R2 receptor is reduced in the presence of the plurality of the compounds, relative to the binding of the compound in the absence of the plurality of compounds, and if the binding is reduced;

15

(d) separately determining the extent of binding to the GABA_BR1/R2 receptor of each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which specifically binds to the GABA_BR1/R2 receptor.

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25

This invention is directed to a method of screening a plurality of chemical compounds not known to bind to a GABA_BR1/R2 receptor to identify a compound which specifically binds to the GABA_BR1/R2 receptor, which comprises

30

(a) contacting a membrane fraction extract from cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with

35

a compound known to bind specifically to the GABA_BR1/R2 receptor;

- 5 (b) contacting the same membrane fraction as in step (a) with the plurality of compounds not known to bind specifically to the GABA_BR1/R2 receptor, under conditions permitting binding of compounds known to bind the GABA_BR1/R2 receptor;
- 10 (c) determining whether the binding of the compound known to bind specifically to the GABA_BR1/R2 receptor is reduced in the presence of the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds, and if the binding is reduced;
- 15 (d) separately determining the extent of binding to the GABA_BR1/R2 receptor of each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which specifically binds to the GABA_BR1/R2 receptor.
- 20
- 25

This invention is directed to a process for determining whether a chemical compound is a GABA_BR1/R2 receptor agonist which comprises contacting cells with the compound under conditions permitting the activation of the GABA_BR1/R2 receptor, and detecting an increase in GABA_BR1/R2 receptor activity, so as to thereby determine whether the compound is a GABA_BR1/R2 receptor agonist.

30

This invention is directed to a process for determining whether a chemical compound is a GABA_BR1/R2 receptor antagonist which comprises contacting cells containing nucleic acid encoding and expressing on their cell

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surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the compound in the presence of a known GABA_BR1/R2 receptor agonist, under conditions permitting the activation of the GABA_BR1/R2 receptor, and detecting a decrease in GABA_BR1/R2 receptor activity, so as to thereby determine whether the compound is a GABA_BR1/R2 receptor antagonist.

This invention is directed to a process for determining whether a chemical compound activates a GABA_BR1/R2 receptor, which comprises contacting cells producing a second messenger response and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the chemical compound under conditions suitable for activation of the GABA_BR1/R2 receptor, and measuring the second messenger response in the presence and in the absence of the chemical compound, a change in the second messenger response in the presence of the chemical compound indicating that the compound activates the GABA_BR1/R2 receptor.

This invention is directed to a process for determining whether a chemical compound inhibits activation of a GABA_BR1/R2 receptor, which comprises separately contacting cells producing a second messenger response and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with both the chemical compound and a second chemical compound known to activate the GABA_BR1/R2 receptor, and with only the second chemical compound, under conditions suitable for activation of the GABA_BR1/R2 receptor, and measuring the second messenger response in the presence of only the second chemical compound and in the presence of both the second chemical compound and the chemical compound, a

smaller change in the second messenger response in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound indicating that the chemical compound inhibits activation of the GABA_BR1/R2 receptor.

This invention is directed to a method of screening a plurality of chemical compounds not known to activate a GABA_BR1/R2 receptor to identify a compound which activates the GABA_BR1/R2 receptor which comprises:

- (a) contacting cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the plurality of compounds not known to activate the GABA_BR1/R2 receptor, under conditions permitting activation of the GABA_BR1/R2 receptor;
- (b) determining whether the activity of the GABA_BR1/R2 receptor is increased in the presence of the compounds, and if it is increased;
- (c) separately determining whether the activation of the GABA_BR1/R2 receptor is increased by each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which activates the GABA_BR1/R2 receptor.

This invention is directed to a method of screening a plurality of chemical compounds not known to inhibit the activation of a GABA_BR1/R2 receptor to identify a compound which inhibits the activation of the GABA_BR1/R2

receptor, which comprises:

- 5 (a) contacting cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the plurality of compounds in the presence of a known GABA_BR1/R2 receptor agonist, under conditions permitting
- 10 activation of the GABA_BR1/R2 receptor;
- 15 (b) determining whether the activation of the GABA_BR1/R2 receptor is reduced in the presence of the plurality of compounds, relative to the activation of the GABA_BR1/R2 receptor in the absence of the plurality of compounds, and if it is reduced;
- 20 (c) separately determining the inhibition of activation of the GABA_BR1/R2 receptor for each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such a plurality of compounds which inhibits the
- 25 activation of the GABA_BR1/R2 receptor.

30 This invention is directed to a process for determining whether a chemical compound is a GABA_BR1/R2 receptor agonist, which comprises preparing a membrane fraction from cells which comprise nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, separately contacting the

35 membrane fraction with both the chemical compound and GTPγS, and with only GTPγS, under conditions permitting the activation of the GABA_BR1/R2 receptor, and detecting GTPγS binding to the membrane fraction, an increase in

GTP γ S binding in the presence of the compound indicating that the chemical compound activates the GABA $_B$ R1/R2 receptor.

5 This invention is directed to a process for determining whether a chemical compound is a GABA $_B$ R1/R2 receptor antagonist, which comprises preparing a membrane fraction from cells which comprise nucleic acid encoding and expressing on their cell surface the
10 GABA $_B$ R1/R2 receptor, wherein such cells do not normally express the GABA $_B$ R1/R2 receptor, separately contacting the membrane fraction with the chemical compound, GTP γ S and a second chemical compound known to activate the GABA $_B$ R1/R2 receptor, with GTP γ S and only the second
15 compound, and with GTP γ S alone, under conditions permitting the activation of the GABA $_B$ R1/R2 receptor, detecting GTP γ S binding to each membrane fraction, and comparing the increase in GTP γ S binding in the presence of the compound and the second compound relative to the
20 binding of GTP γ S alone, to the increase in GTP γ S binding in the presence of the second chemical compound known to activate the GABA $_B$ R1/R2 receptor relative to the binding of GTP γ S alone, a smaller increase in GTP γ S binding in the presence of the compound and the second
25 compound indicating that the compound is a GABA $_B$ R1/R2 receptor antagonist.

30 This invention is directed to a method of treating spasticity in a subject which comprises administering to the subject an amount of a compound which is an agonist of a GABA $_B$ R1/R2 receptor effective to treat spasticity in the subject.

35 This invention is directed to a method of treating asthma in a subject which comprises administering to the subject an amount of a compound which is a GABA $_B$ R1/R2 receptor agonist effective to treat asthma in

the subject.

This invention is directed to a method of treating
incontinence in a subject which comprises administering
5 to the subject an amount of a compound which is a
GABA_BR1/R2 receptor agonist effective to treat
incontinence in the subject.

This invention is directed to a method of decreasing
10 nociception in a subject which comprises administering
to the subject an amount of a compound which is a
GABA_BR1/R2 receptor agonist effective to decrease
nociception in the subject.

This invention is directed to a use of a GABA_BR2 agonist
15 as an antitussive agent which comprises administering
to the subject an amount of a compound which is a
GABA_BR1/R2 receptor agonist effective as an antitussive
agent in the subject.

This invention is directed to a method of treating drug
20 addiction in a subject which comprises administering to
the subject an amount of a compound which is a
GABA_BR1/R2 receptor agonist effective to treat drug
25 addiction in the subject.

This invention is directed to a method of treating
Alzheimer's disease in a subject which comprises
30 administering to the subject an amount of a compound
which is a GABA_BR1/R2 receptor antagonist effective to
treat Alzheimer's disease in the subject.

This invention is directed to a peptide selected from
the group consisting of:

- 35 a) P L Y S I L S A L T I L G M I M A S A F L F F N
I K N;
b) L I I L G G M L S Y A S I F L F G L D G S F V S

E K T;

c) C T V R T W I L T V G Y T T A F G A M F A K T W
R;

d) Q K L L V I V G G M L L I D L C I L I C W Q;

5 e) M T I W L G I V Y A Y K G L L M L F G C F L A
W;

f) A L N D S K Y I G M S V Y N V G I M C I I G A A
V; and

10 g) C I V A L V I I F C S T I T L C L V F V P K L I
T L R T N .

This invention is directed to a compound that prevents
the formation of a GABA_BR1/R2 receptor complex.

15 Finally, this invention provides a process for making a
composition of matter which specifically binds to a
GABA_BR1/R2 receptor which comprises identifying a
chemical compound using any of the processes described
herein for identifying a compound which binds to and/or
20 activates or inhibits activation of a GABA_BR1/R2
receptor and then synthesizing the chemical compound or
a novel structural and functional analog or homolog
thereof. This invention further provides a process for
preparing a pharmaceutical composition which comprises
25 admixing a pharmaceutically acceptable carrier and a
pharmaceutically acceptable amount of a chemical
compound identified by any of the processes described
herein for identifying a compound which binds to and/or
activates or inhibits activation of a GABA_BR1/R2
30 receptor or a novel structural and functional analog or
homolog thereof.

BRIEF DESCRIPTION OF THE FIGURES

Figures 1A-1E Nucleotide coding sequence of the human GABA_BR2 polypeptide (Seq. ID No. 1), with partial 5' and 3' untranslated sequences. Two possible start (ATG) codons are underlined as well as the stop codon (TAA).

Figures 2A-2D Deduced amino acid sequence of the human GABA_BR2 polypeptide (Seq. ID No. 2) encoded by the nucleotide sequence shown in Figures 1A-1E.

Figures 3A-3D Nucleotide coding sequence of the rat GABA_BR2 polypeptide (Seq. ID No. 3). Start (ATG) and stop (TAG) codons are underlined.

Figures 4A-4D Deduced amino acid sequence of the rat GABA_BR2 polypeptide (Seq. ID No. 4) encoded by the nucleotide sequence shown in Figures 3A-3D.

Figures 5A-5D Amino acid sequence of the human GABA_BR2 polypeptide (Seq. ID No. 2) with brackets above the sequence showing the boundaries of seven (7) putative transmembrane domains, numbered consecutively from I to VII.

Figures 6A-6B. Measurement of EC₅₀ for GABA in a cumulative concentration response assay in oocytes expressing GABA_BR1b/GABA_BR2 + GIRKs. Figure 6A: Electrophysiological trace from a voltage clamped oocyte showing increasing inward currents evoked successively by concentrations of GABA ranging from 0.03 to 30 μ M. Numbers over bars indicate concentration of GABA in μ M. hK is 49 mM external K⁺. Figure 6B: Averaged responses from 3-6 oocytes plotted vs. concentration of GABA results in an EC₅₀ value of 1.76 μ M. For each oocyte, currents were normalized to the maximum response at 30 μ M.

Figure 7. Concentration response relationship for baclofen in oocytes expressing GABA_BR1b/GABA_BR2 + GIRKs. Methods are as described for Figure 6.

5 **Figure 8.** Current voltage relationship for the current
activated by GABA in oocytes expressing GABA_BR1b/GABA_BR2
+ GIRKs. Voltage ramps (50 mV/s) from -140 to +40 mV
were applied in the presence of GABA (in hK) and again
10 in the presence of GABA + 100 μ M Ba⁺⁺ to block inward
rectifier current. The resulting traces were
subtracted (GABA alone - GABA + Ba⁺⁺) to yield the Ba⁺⁺-
sensitive portion of the GABA-stimulated current. As
expected for GIRK current, the current displays steep
15 inward rectification and reverses near the predicted
equilibrium potential for K⁺ (-23 mV in hK).

Figures 9A-9B. Electrophysiological responses under
voltage clamp conditions to GABA in an HEK-293 cell
transiently transfected with GABA_BR1b/GABA_BR2 + GIRKs.
20 A) The continuous trace (in presence of 25 mM K⁺) shows
a small constitutive inward rectifier current that is
blocked by Ba⁺⁺ (100 μ M), and a much larger inward
current induced by application of GABA that is also
blocked by Ba⁺⁺. A second GABA-evoked current is
25 abolished by the selective antagonist CGP55845. After
a 1 minute wash period GABA-responsivity returns. B)
Concentration response relation for GABA in 5 HEK-293
cells expressing GABA_BR1b/GABA_BR2 + GIRKs. (See Figure
6B for details.)

30

Figure 10. Alignment of amino acid s predicted for
rat GABA_BR2 and rat GABA_BR1. Horizontal bars indicate
TM regions.

35 **Figures 11A-11D.** Photomicrographs showing the regional
distribution of the GABA_BR1 (A,C) and GABA_BR2 (B,D)
mRNAs in representative coronal rat brain sections.

response curve (solid circle) caused by CGP55845 at 50 nM (open triangle) and CGP54626 at 5 μ M (open circle). Each point is the average response from 4-6 oocytes.

Figure 16. Microphysiometric response to baclofen (100 μ M) from CHO cells expressing combinations of GABA_BR1 and GABA_BR2 (n = 4).

Figures 17A-17D. Co-localization of GABA_BR1 and GABA_BR2 in HEK293 cells by dual wavelength scanning confocal microscopy. Figure 17A: Green channel showing GABA_BR1^{RGS6xH} (labeled with FITC) in cell expressing both GABA_BR1^{RGS6xH} and GABA_BR2^{HA}. Figure 17B: Red channel showing GABA_BR2^{HA} (labeled with TRITC) localization in the same cell. Figure 17C: Dual channel image of the same cell reveals a predominant yellow hue caused by the co-localization of fluorescent tags for GABA_BR1^{RGS6xH} and GABA_BR2^{HA}. Figure 17D: Dual wavelength image of cell expressing GABA_BR2^{HA} (red) and NPY Y5^{Flag} (green). Note the low degree of spatial overlap of the two polypeptides.

Figures 18A-18C. Identification of GABA_BR1 and GABA_BR2 in cell lysates and immunoprecipitates. Figure 18A: Detection of GABA_BR1^{RGS6xH} in whole cell extracts from cells expressing either or both polypeptides. Proteins labeled with anti-His or anti-HA, migrate as monomeric and dimeric forms. Figure 18B: Detection of GABA_BR2^{HA} in whole cell extracts from cells expressing either or both. Labels over lanes denote which polypeptides were transfected. Proteins labeled with anti-His or anti-HA, migrate as monomeric and dimeric forms. Figure 18C: Co-immunoprecipitation of GABA_BR1^{RGS6xH} and GABA_BR2^{HA}. Various transfected cells were immunoprecipitated (IP) with anti-HA or anti-His antibodies, subjected to SDS-PAGE, blotted, and probed for the presence of the HA epitope. Note that in anti-His immunoprecipitated

material, HA immunoreactivity appears only in the lane from cells expressing both proteins.

Figure 19. Rostro-caudal distribution of the GABA_BR2 mRNA in coronal rat brain sections (A-F) and spinal cord (G). Brightfield photomicrographs of the dorsal root (H) and trigeminal (I) ganglia showing silver grains over the cells indicating the presence of GABA_BR2 mRNA.

Figure 20. (A) Detection of Na⁺/K⁺ ATPase by anti-alpha 1 subunit antibodies in membrane fractions enriched in (P1+) or depleted of (P2) plasma membranes (50 :g protein/lane). (B) Co-immunoprecipitation of GABA_BR1^{RGS6xH} and GABA_BR2^{HA} from solubilized P1+ membrane fractions. Note that in anti-His immunoprecipitated material, HA immunoreactivity appears only in the lane from cells expressing both proteins. (C) Western blot showing enrichment of GABA_BR2^{HA} in P1+ membrane fraction as compared to the P2 fraction.

Figure 21. Photomicrographs showing the regional distribution of GABA_BR2 (A,C) and GABA_BR1b (B,D) mRNAs in pairs of adjacent coronal rat brain sections. Arrow heads identify Purkinje cell layer in cerebellum (A,B). High magnification views of hippocampal CA3 region showing both transcripts in cells from alternate sections (C,D). Arrows mark individual cells. Hybridization of GABA_BR2 (E) and GABA_BR1b (F) transcripts in large cells of mesencephalic trigeminal nucleus.

Figure 22A-22D Nucleotide coding sequence of the human GABA_BR2 polypeptide (Seq. ID No. 46). Start (ATG) and stop (TAA) codons are underlined.

Figure 23A-23D Deduced amino acid sequence of the human GABA_BR2 polypeptide (Seq. ID No. 47) encoded by the

DETAILED DESCRIPTION OF THE INVENTION

In this application, the following standard abbreviations are used to indicate specific nucleotide bases:

C = cytosine	A = adenine
T = thymine	G = guanine

In this application, the term 7-TM spanning protein or a 7-TM protein indicates a protein presumed to have seven transmembrane regions which cross the cellular membrane band on its amino acid sequence.

This invention is directed to an isolated nucleic acid encoding a GABA_BR2 polypeptide.

In one embodiment, the nucleic acid is DNA. In another embodiment, the DNA is cDNA. In another embodiment, the DNA is genomic DNA. In another embodiment, the nucleic acid is RNA. In another embodiment, the nucleic acid encodes a mammalian GABA_BR2 polypeptide. In another embodiment, the nucleic acid encodes a rat GABA_BR2 polypeptide. In another embodiment, the nucleic acid encodes a human GABA_BR2 polypeptide.

In another embodiment, the nucleic acid encodes a polypeptide characterized by an amino acid sequence in the transmembrane regions which has an identity of 90% or higher to the amino acid sequence in the transmembrane regions of the human GABA_BR2 polypeptide shown in Figures 5A-5D.

In another embodiment, the nucleic acid encodes a mammalian GABA_BR2 polypeptide which has substantially the same amino acid sequence as does the GABA_BR2 polypeptide encoded by the plasmid BO-55 (ATCC

Accession No. 209104). In another embodiment, the nucleic acid encodes a rat GABA_BR2 polypeptide which has an amino acid sequence encoded by the plasmid BO-55 (ATCC Accession No. 209104).

5

In another embodiment, the nucleic acid encodes a rat GABA_BR2 polypeptide having substantially the same amino acid sequence as the amino acid sequence shown in Figures 4A-4D (Seq. ID No. 4). In another embodiment, the nucleic acid encodes a rat GABA_BR2 polypeptide having the amino acid sequence shown in Figures 4A-4D (Seq. ID No. 4).

10

In another embodiment, the nucleic acid encodes a mammalian GABA_BR2 polypeptide which has substantially the same amino acid sequence as does the GABA_BR2 polypeptide encoded by the plasmid pEXJT3T7-hGABAB2 (ATCC Accession No.). In another embodiment, the nucleic acid encodes a human GABA_BR2 polypeptide which has an amino acid sequence encoded by the plasmid pEXJT3T7-hGABAB2 (ATCC Accession No.).

15

20

In another embodiment, the human GABA_BR2 polypeptide has a sequence, which sequence comprises substantially the same amino acid sequence as the sequence shown in Figures 23A-23D (Seq. ID No. 47).

25

In another embodiment, the human GABA_BR2 polypeptide has a sequence, which sequence comprises the sequence shown in Figures 23A-23D (Seq. ID No. 47).

30

This application further supports an isolated nucleic acid encoding a GABA_BR2 polypeptide, the amino acid sequence of which is encoded by the nucleotide sequence set forth in either the Figures 22A-22D and 3A-3D.

35

Further, the human GABA_BR2 polypeptide described herein

exhibits 38% amino acid identity with the GABA_BR1a polypeptide, while the rat GABA_BR2 polypeptide described herein exhibits 98% identity with the human GABA_BR2 polypeptide.

5

The ATG encoding the methionine at position 16 is surrounded by flanking sequences which correspond to the well-known Kozak consensus sequence for translation initiation (Kozak, 1989 and Kozak, 1991), thus the sequence from amino acid 16 through amino acid 898 is believed to be the most likely polypeptide expressed by the nucleic acid. Neither the ATG encoding methionine 1 nor the ATG encoding methionine 19 has the Kozak flanking sequences; however, it is to be understood that the present invention provides a GABA_BR2 polypeptide having any one of the three possible starting methionines.

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This invention provides a splice variant of the polypeptides disclosed herein. This invention further provides for alternate translation initiation sites and alternately spliced or edited variants of nucleic acids encoding rat and human polypeptides of this invention.

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Methods for production and manipulation of nucleic acid molecules are well known in the art.

30

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This invention also encompasses DNAs and cDNAs which encode amino acid sequences which differ from those of the polypeptides of this invention, but which should not produce phenotypic changes. Alternatively, this invention also encompasses DNAs, cDNAs, and RNAs which hybridize to the DNA, cDNA, and RNA of the subject invention. Hybridization methods are well known to those of skill in the art.

The nucleic acids of the subject invention also include

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nucleic acid molecules coding for polypeptide analogs, fragments or derivatives of antigenic polypeptides which differ from naturally-occurring forms in terms of the identity or location of one or more amino acid residues (deletion analogs containing less than all of the residues specified for the protein, substitution analogs wherein one or more residues specified are replaced by other residues and addition analogs where in one or more amino acid residues is added to a terminal or medial portion of the polypeptides) and which share some or all properties of naturally-occurring forms. These molecules include: the incorporation of codons "preferred" for expression by selected non-mammalian hosts; the provision of sites for cleavage by restriction endonuclease enzymes; and the provision of additional initial, terminal or intermediate DNA sequences that facilitate construction of readily expressed vectors.

The modified polypeptides of this invention may be transfected into cells either transiently or stably using methods well-known in the art, examples of which are disclosed herein.

This invention also provides for binding assays using the modified polypeptides, in which the polypeptide is expressed either transiently or in stable cell lines. This invention further provides for a compound identified using a modified polypeptide in a binding assay such as the binding assays described herein.

The nucleic acids described and claimed herein are useful for the information which they provide concerning the amino acid sequence of the polypeptide and as products for the large scale synthesis of the polypeptide by a variety of recombinant techniques. The nucleic acid molecule is useful for generating new

cloning and expression vectors, transformed and
transfected prokaryotic and eukaryotic host cells, and
new and useful methods for cultured growth of such host
cells capable of expression of the polypeptide and
related products.

Vectors which comprise the isolated nucleic acid
molecule described hereinabove also are provided.
Suitable vectors comprise, but are not limited to, a
plasmid or a virus. These vectors may be transformed
into a suitable host cell to form a host cell
expression system for the production of a GABA_BR2
polypeptide. Suitable host cells include, for example,
neuronal cells such as the glial cell line C6, a
Xenopus cell such as an oocyte or melanophore cell, as
well as numerous mammalian cells and non-neuronal
cells.

This invention further provides for any vector or
plasmid which comprises modified untranslated
sequences, which are beneficial for expression in
desired host cells or for use in binding or functional
assays. For example, a vector or plasmid with
untranslated sequences of varying lengths may express
differing amounts of the polypeptide depending upon the
host cell used. In an embodiment, the vector or
plasmid comprises the coding sequence of the
polypeptide and the regulatory elements necessary for
expression in the host cell.

As used herein, the phrase "specifically hybridizing"
means the ability of a nucleic acid molecule to
recognize a nucleic acid sequence complementary to its
own and to form double-helical segments through
hydrogen bonding between complementary base pairs. The
term "complementary" is used in its usual sense in the
art, i.e., G and C are complementary and A is

complementary to T (or U in RNA), such that two strands of nucleic acid are "complementary" only if every base matches the opposing base exactly.

5 This invention is directed to a purified GABA_BR2 protein.

This invention is directed to a vector comprising a above-identified nucleic acid.

10 In one embodiment, the vector is adapted for expression in a bacterial cell which comprises the regulatory elements necessary for expression of the nucleic acid in the bacterial cell operatively linked to the nucleic acid encoding a GABA_BR2 polypeptide so as to permit
15 expression thereof.

In another embodiment, the vector is adapted for expression in an amphibian cell which comprises the regulatory elements necessary for expression of the
20 nucleic acid in the amphibian cell operatively linked to the nucleic acid encoding a GABA_BR2 polypeptide so as to permit expression thereof.

25 In another embodiment, the vector is adapted for expression in a yeast cell which comprises the regulatory elements necessary for expression of the nucleic acid in the yeast cell operatively linked to the nucleic acid encoding a GABA_BR2 polypeptide so as to
30 permit expression thereof.

In another embodiment, the vector is adapted for expression in an insect cell which comprises the regulatory elements necessary for expression of the
35 nucleic acid in the insect cell operatively linked to the nucleic acid encoding the GABA_BR2 polypeptide so as to permit expression thereof.

In one embodiment, the vector is a baculovirus.

In another embodiment, the vector is adapted for expression in a mammalian cell which comprises the regulatory elements necessary for expression of the nucleic acid in the mammalian cell operatively linked to the nucleic acid encoding a GABA_BR2 polypeptide so as to permit expression thereof.

In one embodiment, the vector is a plasmid.

In a further embodiment, the plasmid is designated BO-55 (ATCC Accession No. 209104).

In a further embodiment, the plasmid is designated pEXJT3T7-hGABAB2 (ATCC Accession No.).

This invention provides a plasmid designated pEXJT3T7-hGABAB2 (ATCC Accession No.) which comprises the regulatory elements necessary for expression of DNA in a mammalian cell operatively linked to DNA encoding the human polypeptide so as to permit expression thereof.

This plasmid (pEXJT3T7-hGABAB2) was deposited on December 9, 1998, with the American Type Culture Collection (ATCC), 10801 University Blvd., Manassas, Virginia 20110-2209, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and was accorded ATCC Accession No.

This invention provides a plasmid designated BO-55 (ATCC Accession No. 209104) which comprises the regulatory elements necessary for expression of DNA in a mammalian cell operatively linked to DNA encoding the rat polypeptide so as to permit expression thereof.

This plasmid (BO-55) was deposited on June 10, 1997, with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and was accorded ATCC Accession No. 209104.

Nucleic acid probe technology is well known to those skilled in the art who will readily appreciate that such probes may vary greatly in length and may be labeled with a detectable label, such as a radioisotope or fluorescent dye, to facilitate detection of the probe. DNA probe molecules may be produced by insertion of a DNA molecule which encodes the polypeptides of this invention into suitable vectors, such as plasmids or bacteriophages, followed by transforming into suitable bacterial host cells, replication in the transformed bacterial host cells and harvesting of the DNA probes, using methods well known in the art. Alternatively, probes may be generated chemically from DNA synthesizers.

RNA probes may be generated by inserting the DNA molecule which encodes the polypeptides of this invention downstream of a bacteriophage promoter such as T3, T7 or SP6. Large amounts of RNA probe may be produced by incubating the labeled nucleotides with the linearized fragment where it contains an upstream promoter in the presence of the appropriate RNA polymerase.

This invention is directed to a method of detecting a nucleic acid encoding a GABA_BR2 polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding

the GABA_BR2 polypeptide, wherein the probe has a unique sequence, which sequence is present within one of the two strands of the nucleic acid encoding the GABA_BR2 polypeptide contained in plasmid BO-55, and detecting hybridization of the probe to the nucleic acid.

This invention is directed to a method of detecting a nucleic acid encoding a GABA_BR2 polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the GABA_BR2 polypeptide, wherein the probe has a unique sequence, which sequence is present within (a) the nucleic acid sequence shown in Figures 22A-22D (Seq. ID No. 46) or (b) the reverse complement to the nucleic acid sequence shown in Figures 22A-22D (Seq. ID No. 46), and detecting hybridization of the probe to the nucleic acid.

This invention is directed to a method of detecting a nucleic acid encoding a GABA_BR2 polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the GABA_BR2 polypeptide, wherein the probe has a unique sequence, which sequence is present within one of the two strands of the nucleic acid encoding the GABA_BR2 polypeptide contained in plasmid pEXT3T7-hGABAB2 and detecting hybridization of the probe to the nucleic acid.

This invention is directed to a method of detecting a nucleic acid encoding a GABA_BR2 polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the GABA_BR2 polypeptide, wherein the probe has a unique

sequence, which sequence is present within (a) the
nucleic acid sequence shown in Figures 3A-3D (Seq. ID
No. 3) or (b) the reverse complement to the nucleic
acid sequence shown in Figures 3A-3D (Seq. ID No. 3),
and detecting hybridization of the probe to the nucleic
acid.

In one embodiment, the nucleic acid is DNA.

In another embodiment, the nucleic acid is RNA.

In one embodiment, the probe comprises at least 15
nucleotides complementary to a unique segment of the
sequence of the nucleic acid molecule encoding the
GABA_BR2 polypeptide.

This invention is directed to a method of detecting a
nucleic acid encoding a GABA_BR2 polypeptide, which
comprises contacting the nucleic acid with a probe
comprising a nucleic acid of at least 15 nucleotides
which is complementary to the antisense sequence of a
unique segment of the sequence of the nucleic acid
encoding the GABA_BR2 polypeptide, and detecting
hybridization of the probe to the nucleic acid.

This invention is directed to a method of inhibiting
translation of mRNA encoding a GABA_BR2 polypeptide
which comprises contacting such mRNA with an antisense
oligonucleotide having a sequence capable of
specifically hybridizing to the above-identified mRNA,
so as to prevent translation of the mRNA.

This invention is directed to a method of inhibiting
translation of mRNA encoding a GABA_BR2 polypeptide
which comprises contacting such mRNA with an antisense
oligonucleotide having a sequence capable of
specifically hybridizing to the above-identified

genomic DNA.

In one embodiment, the oligonucleotide comprises
chemically modified nucleotides or nucleotide
analogues.

In another embodiment, the isolated antibody is capable
of binding to a GABA_BR2 polypeptide encoded by an above-
identified nucleic acid.

In another embodiment, the GABA_BR2 polypeptide is a
human GABA_BR2 polypeptide.

This invention is directed to an antibody capable of
competitively inhibiting the binding of an above-
identified antibody to a GABA_BR2 polypeptide.

In one embodiment, the antibody is a monoclonal
antibody.

In one embodiment, the monoclonal antibody is directed
to an epitope of a GABA_BR2 polypeptide present on the
surface of a GABA_BR2 polypeptide expressing cell.

In another embodiment, the oligonucleotide is coupled
to a substance which inactivates mRNA.

In another embodiment, the substance which inactivates
mRNA is a ribozyme.

This invention is directed to a pharmaceutical
composition which comprises an amount of an above-
identified antibody effective to block binding of a
ligand to the GABA_BR2 polypeptide and a pharmaceutically
acceptable carrier.

As used herein, "pharmaceutically acceptable carriers"

means any of the standard pharmaceutically acceptable carriers. Examples include, but are not limited to, phosphate buffered saline, physiological saline, water and emulsions, such as oil/water emulsions.

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Animal model systems which elucidate the physiological and behavioral roles of the polypeptides of this invention are produced by creating transgenic animals in which the activity of the polypeptide is either increased or decreased, or the amino acid sequence of the expressed polypeptide is altered, by a variety of techniques. Examples of these techniques include, but are not limited to: 1) Insertion of normal or mutant versions of DNA encoding the polypeptide, by microinjection, electroporation, retroviral transfection or other means well known to those skilled in the art, into appropriate fertilized embryos in order to produce a transgenic animal or 2) Homologous recombination of mutant or normal, human or animal versions of these genes with the native gene locus in transgenic animals to alter the regulation of expression or the structure of these polypeptide sequences. The technique of homologous recombination is well known in the art. It replaces the native gene with the inserted gene and so is useful for producing an animal that cannot express native polypeptides but does express, for example, an inserted mutant polypeptide, which has replaced the native polypeptide in the animal's genome by recombination, resulting in underexpression of the transporter. Microinjection adds genes to the genome, but does not remove them, and so is useful for producing an animal which expresses its own and added polypeptides, resulting in overexpression of the polypeptides.

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One means available for producing a transgenic animal, with a mouse as an example, is as follows: Female mice

are mated, and the resulting fertilized eggs are dissected out of their oviducts. The eggs are stored in an appropriate medium such as M2 medium. DNA or cDNA encoding a polypeptide of this invention is purified from a vector by methods well known in the art. Inducible promoters may be fused with the coding region of the DNA to provide an experimental means to regulate expression of the trans-gene. Alternatively, or in addition, tissue specific regulatory elements may be fused with the coding region to permit tissue-specific expression of the trans-gene. The DNA, in an appropriately buffered solution, is put into a microinjection needle (which may be made from capillary tubing using a pipet puller) and the egg to be injected is put in a depression slide. The needle is inserted into the pronucleus of the egg, and the DNA solution is injected. The injected egg is then transferred into the oviduct of a pseudopregnant mouse (a mouse stimulated by the appropriate hormones to maintain pregnancy but which is not actually pregnant), where it proceeds to the uterus, implants, and develops to term. As noted above, microinjection is not the only method for inserting DNA into the egg cell, and is used here only for exemplary purposes.

This invention is directed to a transgenic, nonhuman mammal expressing DNA encoding a GABA_BR2 polypeptide.

This invention is directed to a transgenic, nonhuman mammal comprising a homologous recombination knockout of the native GABA_BR2 polypeptide.

This invention is further directed to a transgenic, nonhuman mammal whose genome comprises antisense DNA complementary to DNA encoding a GABA_BR2 polypeptide so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding such GABA_BR2

polypeptide and which hybridizes to such mRNA encoding such GABA_BR2 polypeptide, thereby reducing its translation.

5 This invention is directed to an above-identified transgenic, nonhuman mammal, wherein the DNA encoding the GABA_BR2 polypeptide additionally comprises an inducible promoter.

10 This invention is directed to an above-identified transgenic, nonhuman mammal, wherein the DNA encoding the GABA_BR2 polypeptide additionally comprises tissue specific regulatory elements.

15 This invention is directed to an above-identified transgenic, nonhuman mammal, wherein the transgenic, nonhuman mammal is a mouse.

20 This invention is directed to method of detecting the presence of a GABA_BR2 polypeptide on the surface of a cell which comprises contacting the cell with an above-identified antibody under conditions permitting binding of the antibody to the polypeptide, detecting the presence of the antibody bound to the cell, and thereby
25 detecting the presence of a GABA_BR2 polypeptide on the surface of the cell.

This invention is directed to a method of preparing a purified GABA_BR2 polypeptide which comprises:

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a. inducing cells to express a GABA_BR2 polypeptide;

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b. recovering the polypeptide so expressed from the induced cells; and

c. purifying the polypeptide so recovered.

This invention is directed to a method of preparing the purified GABA_BR2 polypeptide which comprises:

- 5 a. inserting a nucleic acid encoding the GABA_BR2 polypeptide into a suitable vector;
- b. introducing the resulting vector in a suitable host cell;
- 10 c. placing the resulting cell in suitable condition permitting the production of the GABA_BR2 polypeptide;
- d. recovering the polypeptide produced by the resulting cell; and
- 15 e. isolating or purifying the polypeptide so recovered.

20 This invention is directed to a GABA_BR1/R2 receptor comprising two polypeptides, one of which is a GABA_BR2 polypeptide and another of which is a GABA_BR1 polypeptide.

25 This invention is directed to a method of forming a GABA_BR1/R2 receptor which comprises inducing cells to express both a GABA_BR1 polypeptide and a GABA_BR2 polypeptide.

30 GABA_BR1 as used in this application could be GABA_BR1a or GABA_BR1b. The observation that at least two variants of the GABA_BR1 polypeptide exist raises the possibility that GABA_BR2 splice variants may exist or that there may exist introns in coding or non-coding regions of the
35 genes encoding the GABA_BR2 polypeptides. In addition, spliced form(s) of mRNA may encode additional amino acids either upstream of the currently defined starting

methionine or within the coding region. Further, the
existence and use of alternative exons is possible,
whereby the mRNA may encode different amino acids
within the region comprising the exon. In addition,
5 single amino acid substitutions may arise via the
mechanism of RNA editing such that the amino acid
sequence of the expressed protein is different than
that encoded by the original gene (Burns et al., 1996;
Chu et al., 1996). Such variants may exhibit
10 pharmacologic properties differing from the polypeptide
encoded by the original gene.

The activity of a G-protein coupled receptor (GPCR)
typically is measured using any of a variety of
15 functional assays in which activation of the receptor
in question results in an observable change in the
level of some second messenger system, including but
not limited to adenylate cyclase, calcium mobilization,
arachidonic acid release, ion channel activity,
20 inositol phospholipid hydrolysis or guanylyl cyclase.
Heterologous expression systems utilizing appropriate
host cells to express the nucleic acids of the subject
invention are used to obtain the desired second
messenger coupling. Receptor activity may also be
25 assayed in an oocyte expression system.

The pharmacologic properties of the receptor described
herein when GABA_BR2 is co-expressed with GABA_BR1, are
similar to the pharmacologic properties of the GABA_B
30 receptor observed using tissues. For convenience, in
the context of the present invention applicants will
refer to the product of the heterologous coexpression
of GABA_BR2 and GABA_BR1 as the "GABA_BR1/R2 receptor."
Thus, a cell expressing nucleic acid encoding a
35 GABA_BR1/R2 receptor is to be understood to refer to a
cell expressing both nucleic acid encoding a GABA_BR1
polypeptide and nucleic acid encoding a GABA_BR2

polypeptide. In this application, GABA_BR1 can be either GABA_BR1a or GABA_BR1b.

5 This invention is directed to an antibody capable of binding to a GABA_BR1/R2 receptor, wherein the GABA_BR2 polypeptide is encoded by an above-identified nucleic acid.

10 This invention is directed to an above-identified antibody, wherein the GABA_BR2 polypeptide is a human GABA_BR2 polypeptide.

15 This invention is directed to an antibody capable of competitively inhibiting the binding of an above-identified antibody to a GABA_BR1/R2 receptor.

In one embodiment, the antibody is a monoclonal antibody.

20 This invention is directed to an above-identified monoclonal antibody directed to an epitope of a GABA_BR1/R2 receptor present on the surface of a GABA_BR1/R2 polypeptide expressing cell.

25 This invention is directed to a pharmaceutical composition which comprises an amount of an above-identified antibody effective to block binding of a ligand to the GABA_BR1/R2 receptor and a pharmaceutically acceptable carrier.

30 This invention is directed to a transgenic, nonhuman mammal expressing a GABA_BR1/R2 receptor, which is not naturally expressed by the mammal.

35 This invention is directed to a transgenic, nonhuman mammal comprising a homologous recombination knockout of the native GABA_BR1/R2 receptor.

In one embodiment, the transgenic nonhuman mammal is a mouse.

5 This invention is directed to a method of detecting the presence of a GABA_BR1/R2 receptor on the surface of a cell which comprises contacting the cell with an above-identified antibody under conditions permitting binding of the antibody to the receptor, detecting the presence of the antibody bound to the cell, and thereby
10 detecting the presence of a GABA_BR1/R2 receptor on the surface of the cell.

15 This invention is directed to a method of determining the physiological effects of varying levels of activity of GABA_BR1/R2 receptors which comprises producing an above-identified transgenic nonhuman mammal whose levels of GABA_BR1/R2 receptor activity vary due to the presence of an inducible promoter which regulates GABA_BR1/R2 receptor expression.

20 This invention is directed to a method of determining the physiological effects of varying levels of activity of GABA_BR1/R2 receptors which comprises producing a panel of above-identified transgenic nonhuman mammals,
25 each expressing a different amount of GABA_BR1/R2 receptor.

30 This invention is directed to a method for identifying an antagonist capable of alleviating an abnormality, by decreasing the activity of a GABA_BR1/R2 receptor comprising administering a compound to a above-identified transgenic nonhuman mammal, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic,
35 nonhuman mammal, the alleviation of the abnormality identifying the compound as the antagonist.

This invention is directed to an antagonist identified by an above-identified method.

5 This invention is directed to a pharmaceutical composition comprising an above-identified antagonist and a pharmaceutically acceptable carrier.

10 This invention is directed to a method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a GABA_BR1/R2 receptor which comprises administering to a subject an effective amount of an above-identified pharmaceutical composition, thereby treating the abnormality.

15 This invention is directed to a method for identifying an agonist capable of alleviating an abnormality, by increasing the activity of a GABA_BR1/R2 receptor comprising administering a compound to an above-identified transgenic nonhuman mammal, and determining
20 whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal, the alleviation of the abnormality identifying the compound as the agonist.

25 This invention is directed to an agonist identified by an above-identified method.

30 This invention is directed to a pharmaceutical composition comprising an above-identified agonist and a pharmaceutically acceptable carrier.

35 This invention is directed to a method for treating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a GABA_BR1/R2 receptor which comprises administering to a subject an effective amount of an above-identified pharmaceutical composition, thereby treating the abnormality.

This invention is directed to a cell which expresses on its surface a mammalian GABA_BR1/R2 receptor that is not naturally expressed on the surface of such cell.

5 This invention is directed to a cell, wherein the mammalian GABA_BR1/R2 receptor comprises two polypeptides, one of which is a GABA_BR2 polypeptide and another of which is a GABA_BR1 polypeptide.

10 This invention is directed to a process for identifying a chemical compound which specifically binds to a GABA_BR1/R2 receptor which comprises contacting cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such
15 cells do not normally express the GABA_BR1/R2 receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the GABA_BR1/R2 receptor.

20 This invention is directed to a process for identifying a chemical compound which specifically binds to a GABA_BR1/R2 receptor which comprises contacting a membrane fraction from a cell extract of cells containing nucleic acid encoding and expressing on
25 their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the GABA_BR1/R2 receptor.

30 In one embodiment, the GABA_BR1/R2 receptor is a mammalian GABA_BR1/R2 receptor.

35 In another embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid BO-55 (ATCC Accession No. 209104).

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This invention is directed to a compound identified by an above-identified process.

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In another embodiment, the cell is a mammalian cell.

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In another embodiment, the cell is nonneuronal in origin.

In another embodiment, the nonneuronal cell is a COS-7

cell, 293 human embryonic kidney cell, a CHO cell, a NIH-3T3 cell a mouse Y1 cell or LM(tk-) cell.

5 In another embodiment, the compound is not previously known to bind to a GABA_BR1/R2 receptor.

This invention is directed to a compound identified by an above-identified process.

10 This invention is directed to a process involving competitive binding for identifying a chemical compound which specifically binds to a GABA_BR1/R2 receptor which comprises separately contacting cells expressing on
15 their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific
20 binding of the chemical compound to the GABA_BR1/R2 receptor, a decrease in the binding of the second chemical compound to the GABA_BR1/R2 receptor in the presence of the chemical compound indicating that the chemical compound binds to the GABA_BR1/R2 receptor.

25 This invention is directed to a process involving competitive binding for identifying a chemical compound which specifically binds to a human GABA_BR1/R2 receptor which comprises separately contacting a membrane
30 fraction from a cell extract of cells expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only
35 the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the GABA_BR1/R2

receptor, a decrease in the binding of the second chemical compound to the GABA_BR1/R2 receptor in the presence of the chemical compound indicating that the chemical compound binds to the GABA_BR1/R2 receptor.

5

In one embodiment, the GABA_BR1/R2 receptor is a mammalian GABA_BR1/R2 receptor.

10

In another embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that encoded by plasmid BO-55 (ATCC Accession No. 209104).

15

In another embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that shown in Figures 23A-23D (Seq. ID No. 47).

20

In another embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has the amino acid sequence shown in Figures 23A-23D (Seq. ID No. 47).

25

In another embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that encoded by plasmid pEXJT3T7-hGABAB2 (ATCC Accession No.).

30

In another embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as the sequence shown in Figures 23A-23D (Seq. ID No. 47).

35

In another embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has the sequence shown in Figures 23A-23D (Seq. ID No. 47).

In another embodiment, the cell is an insect cell.

In another embodiment, the cell is a mammalian cell.

In another embodiment, the cell is nonneuronal in origin.

5

In another embodiment, the nonneuronal cell is a COS-7 cell, 293 human embryonic kidney cell, a CHO cell, a NIH-3T3 cell a mouse Y1 cell or LM(tk-) cell.

10

In another embodiment, the compound is not previously known to bind to a GABA_BR1/R2 receptor.

This invention is directed to a compound identified by an above-identified process.

15

This invention is directed to a method of screening a plurality of chemical compounds not known to bind to a GABA_BR1/R2 receptor to identify a compound which specifically binds to the GABA_BR1/R2 receptor, which comprises

20

(a) contacting cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with a compound known to bind specifically to the GABA_BR1/R2 receptor;

25

(b) contacting the same cells as in step (a) with the plurality of compounds not known to bind specifically to the GABA_BR1/R2 receptor, under conditions permitting binding of compounds known to bind the GABA_BR1/R2 receptor;

30

(c) determining whether the binding of the compound known to bind specifically to the GABA_BR1/R2 receptor is reduced in the presence

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of the plurality of the compounds, relative to the binding of the compound in the absence of the plurality of compounds, and if the binding is reduced;

5

- (d) separately determining the extent of binding to the GABA_BR1/R2 receptor of each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which specifically binds to the GABA_BR1/R2 receptor.

10

This invention is directed to a method of screening a plurality of chemical compounds not known to bind to a GABA_BR1/R2 receptor to identify a compound which specifically binds to the GABA_BR1/R2 receptor, which comprises

15

- (a) contacting a membrane fraction extract from cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with a compound known to bind specifically to the GABA_BR1/R2 receptor;

20

- (b) contacting the same membrane fraction as in step (a) with the plurality of compounds not known to bind specifically to the GABA_BR1/R2 receptor, under conditions permitting binding of compounds known to bind the GABA_BR1/R2 receptor;

30

- (c) determining whether the binding of the compound known to bind specifically to the GABA_BR1/R2 receptor is reduced in the presence of the plurality of compounds, relative to

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the binding of the compound in the absence of the plurality of compounds, and if the binding is reduced;

- 5 (d) separately determining the extent of binding to the GABA_BR1/R2 receptor of each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which
10 specifically binds to the GABA_BR1/R2 receptor.

In one embodiment, the GABA_BR1/R2 receptor is a mammalian GABA_BR1/R2 receptor.

15 In one embodiment, the cell is a mammalian cell.

In one embodiment, the mammalian cell is non-neuronal in origin.

20 In one embodiment, the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell, a CHO cell, a mouse Y1 cell or an NIH-3T3 cell.

25 This invention is directed to a process for determining whether a chemical compound is a GABA_BR1/R2 receptor agonist which comprises contacting cells with the compound under conditions permitting the activation of the GABA_BR1/R2 receptor, and detecting an increase in GABA_BR1/R2 receptor activity, so as to thereby determine
30 whether the compound is a GABA_BR1/R2 receptor agonist.

35 This invention is directed to a process for determining whether a chemical compound is a GABA_BR1/R2 receptor antagonist which comprises contacting cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the

compound in the presence of a known GABA_BR1/R2 receptor agonist, under conditions permitting the activation of the GABA_BR1/R2 receptor, and detecting a decrease in GABA_BR1/R2 receptor activity, so as to thereby determine whether the compound is a GABA_BR1/R2 receptor antagonist.

Expression of genes in *Xenopus* oocytes is well known in the art (A. Coleman, Transcription and Translation: A Practical Approach (B.D. Hanes, S.J. Higgins, eds., pp 271-302, IRL Press, Oxford, 1984; Y. Masu et al., Nature **329**:21583-21586, 1994) and is performed using microinjection of native mRNA or in vitro synthesized mRNA into frog oocytes. The preparation of in vitro synthesized mRNA can be performed by various standard techniques (J. Sambrook et al., Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1989) including using T7 polymerase with the mCAP RNA capping kit (Stratagene).

In one embodiment, the cells additionally express nucleic acid encoding GIRK1 and GIRK4.

In another embodiment, the GABA_BR2 receptor is a mammalian GABA_BR2 receptor.

This invention is directed to a pharmaceutical composition which comprises an amount of a GABA_BR1/R2 receptor agonist determined to be an agonist by an above-identified process effective to increase activity of a GABA_BR1/R2 receptor and a pharmaceutically acceptable carrier.

This invention is directed to a pharmaceutical, wherein the GABA_BR1/R2 receptor agonist was not previously known.

This invention is directed to a pharmaceutical composition which comprises an amount of a GABA_BR1/R2 receptor antagonist determined to be an antagonist an above-identified process effective to reduce activity of a GABA_BR1/R2 receptor and a pharmaceutically acceptable carrier.

This invention is directed to a pharmaceutical composition, wherein the GABA_BR1/R2 receptor antagonist was not previously known.

This invention is directed to a process for determining whether a chemical compound activates a GABA_BR1/R2 receptor, which comprises contacting cells producing a second messenger response and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the chemical compound under conditions suitable for activation of the GABA_BR1/R2 receptor, and measuring the second messenger response in the presence and in the absence of the chemical compound, a change in the second messenger response in the presence of the chemical compound indicating that the compound activates the GABA_BR1/R2 receptor.

In one embodiment, the second messenger response comprises potassium channel activation and the change in second messenger is an increase in the level of potassium current.

This invention is directed to a process for determining whether a chemical compound inhibits activation of a GABA_BR1/R2 receptor, which comprises separately contacting cells producing a second messenger response and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with both the

chemical compound and a second chemical compound known to activate the GABA_BR1/R2 receptor, and with only the second chemical compound, under conditions suitable for activation of the GABA_BR1/R2 receptor, and measuring the second messenger response in the presence of only the second chemical compound and in the presence of both the second chemical compound and the chemical compound, a smaller change in the second messenger response in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound indicating that the chemical compound inhibits activation of the GABA_BR1/R2 receptor.

In one embodiment, the second messenger response comprises potassium channel activation and the change in second messenger response is a smaller increase in the level of inward potassium current in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound.

This invention is directed to an above-identified process, wherein the GABA_BR1/R2 receptor is a mammalian GABA_BR1/R2 receptor.

In one embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid BO-55 (ATCC Accession No. 209104).

In another embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that shown in Figures 4A-4D (Seq. ID No. 4).

In another embodiment, the GABA_BR1/R2 receptor comprises

a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that shown in Figures 23A-23D (Seq. ID No. 47).

5 In another embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has the sequence, shown in Figures 23A-23D (Seq. ID No. 47).

10 In another embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid pEXJT3T7-hGABAB2 (ATCC Accession No.).

15 This invention is directed to an above-identified process, wherein the cell is an insect cell.

This invention is directed to an above-identified process, wherein the cell is a mammalian cell.

20 In one embodiment, the mammalian cell is nonneuronal in origin.

25 In another embodiment, the nonneuronal cell is a COS-7 cell, CHO cell, 293 human embryonic kidney cell, NIH-3T3 cell or LM(tk-) cell.

In another embodiment, the compound was not previously known to activate or inhibit a GABA_BR1/R2 receptor.

30 This invention is directed to a compound determined by an above-identified process.

35 This invention is directed to a pharmaceutical composition which comprises an amount of a GABA_BR1/R2 receptor agonist determined by an above-identified process effective to increase activity of a GABA_BR1/R2 receptor and a pharmaceutically acceptable carrier.

In one embodiment, the GABA_BR1/R2 receptor agonist was not previously known.

This invention is directed to a pharmaceutical composition which comprises an amount of a GABA_BR1/R2 receptor antagonist determined by an above-identified process effective to reduce activity of a GABA_BR1/R2 receptor and a pharmaceutically acceptable carrier.

In one embodiment, the GABA_BR1/R2 receptor antagonist was not previously known.

This invention is directed to method of screening a plurality of chemical compounds not known to activate a GABA_BR1/R2 receptor to identify a compound which activates the GABA_BR1/R2 receptor which comprises:

(a) contacting cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the plurality of compounds not known to activate the GABA_BR1/R2 receptor, under conditions permitting activation of the GABA_BR1/R2 receptor;

(b) determining whether the activity of the GABA_BR1/R2 receptor is increased in the presence of the compounds, and if it is increased;

(c) separately determining whether the activation of the GABA_BR1/R2 receptor is increased by each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which activates the

GABA_BR1/R2 receptor.

In one embodiment, the cells express nucleic acid encoding GIRK1 and GIRK4.

5

In another embodiment, the GABA_BR1/R2 receptor is a mammalian GABA_BR1/R2 receptor.

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This invention is directed to a method of screening a plurality of chemical compounds not known to inhibit the activation of a GABA_BR1/R2 receptor to identify a compound which inhibits the activation of the GABA_BR1/R2 receptor, which comprises:

15

(a) contacting cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the plurality of compounds in the presence of a known GABA_BR1/R2 receptor agonist, under conditions permitting activation of the GABA_BR1/R2 receptor;

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25

(b) determining whether the activation of the GABA_BR1/R2 receptor is reduced in the presence of the plurality of compounds, relative to the activation of the GABA_BR1/R2 receptor in the absence of the plurality of compounds, and if it is reduced;

30

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(c) separately determining the inhibition of activation of the GABA_BR1/R2 receptor for each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such a plurality of compounds which inhibits the activation of the GABA_BR1/R2 receptor.

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In one embodiment, the cells express nucleic acid encoding GIRK1 and GIRK4.

5 In one embodiment, the GABA_BR1/R2 receptor is a mammalian GABA_BR1/R2 receptor.

In another embodiment, wherein the cell is a mammalian cell.

10 In another embodiment, the mammalian cell is non-neuronal in origin.

15 In another embodiment, the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell or an NIH-3T3 cell.

20 This invention is directed to a pharmaceutical composition comprising a compound identified by an above-identified method, effective to increase GABA_BR1/R2 receptor activity and a pharmaceutically acceptable carrier.

25 This invention is directed to a pharmaceutical composition comprising a compound identified by an above-identified method, effective to decrease GABA_BR1/R2 receptor activity and a pharmaceutically acceptable carrier.

30 This invention is directed to a process for determining whether a chemical compound is a GABA_BR1/R2 receptor agonist, which comprises preparing a membrane fraction from cells which comprise nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express
35 the GABA_BR1/R2 receptor, separately contacting the membrane fraction with both the chemical compound and GTPγS, and with only GTPγS, under conditions permitting

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the activation of the GABA_BR1/R2 receptor, and detecting
GTPγS binding to the membrane fraction, an increase in
GTPγS binding in the presence of the compound
indicating that the chemical compound activates the
5 GABA_BR1/R2 receptor.

This invention is directed to a process for determining
whether a chemical compound is a GABA_BR1/R2 receptor
antagonist, which comprises preparing a membrane
10 fraction from cells which comprise nucleic acid
encoding and expressing on their cell surface the
GABA_BR1/R2 receptor, wherein such cells do not normally
express the GABA_BR1/R2 receptor, separately contacting
the membrane fraction with the chemical compound, GTPγS
15 and a second chemical compound known to activate the
GABA_BR1/R2 receptor, with GTPγS and only the second
compound, and with GTPγS alone, under conditions
permitting the activation of the GABA_BR1/R2 receptor,
detecting GTPγS binding to each membrane fraction, and
20 comparing the increase in GTPγS binding in the presence
of the compound and the second compound relative to the
binding of GTPγS alone, to the increase in GTPγS
binding in the presence of the second chemical compound
known to activate the GABA_BR1/R2 receptor relative to
25 the binding of GTPγS alone, a smaller increase in GTPγS
binding in the presence of the compound and the second
compound indicating that the compound is a GABA_BR1/R2
receptor antagonist.

30 In one embodiment, the GABA_BR2 receptor is a mammalian
GABA_BR2 receptor.

In another embodiment, the GABA_BR1/R2 receptor comprises
a GABA_BR2 polypeptide which has substantially the same
35 amino acid sequence as that encoded by the plasmid BO-
55 (ATCC Accession No. 209104).

In another embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that shown in Figures 4A-4D (Seq. ID No. 4).

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In another embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid pEXJT3T7-hGABAB2 (ATCC Accession No.).

10

In another embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that shown in Figures 23A-23D (Seq. ID No. 47).

15

In another embodiment, the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has the sequence shown in Figures 23A-23D (Seq. ID No. 47).

20

In another embodiment, the cell is an insect cell.

In another embodiment, the cell is a mammalian cell.

25

In another embodiment, the mammalian cell is nonneuronal in origin.

30

In another embodiment, the nonneuronal cell is a COS-7 cell, CHO cell, 293 human embryonic kidney cell, NIH-3T3 cell or LM(tk-) cell.

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In another embodiment, the compound was not previously known to be an agonist or antagonist of a GABA_BR1/R2 receptor.

This invention is directed to a compound determined to be an agonist or antagonist of a GABA_BR1/R2 receptor by an above-identified process.

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This invention is directed to a method of treating spasticity in a subject which comprises administering to the subject an amount of a compound which is an agonist of a GABA_BR1/R2 receptor effective to treat spasticity in the subject.

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This invention is directed to a method of treating asthma in a subject which comprises administering to the subject an amount of a compound which is a GABA_BR1/R2 receptor agonist effective to treat asthma in the subject.

10

This invention is directed to a method of treating incontinence in a subject which comprises administering to the subject an amount of a compound which is a GABA_BR1/R2 receptor agonist effective to treat incontinence in the subject.

15

This invention is directed to method of decreasing nociception in a subject which comprises administering to the subject an amount of a compound which is a GABA_BR1/R2 receptor agonist effective to decrease nociception in the subject.

20

This invention is directed to a use of a GABA_BR2 agonist as an antitussive agent which comprises administering to the subject an amount of a compound which is a GABA_BR1/R2 receptor agonist effective as an antitussive agent in the subject.

25

This invention is directed to a method of treating drug addiction in a subject which comprises administering to the subject an amount of a compound which is a GABA_BR1/R2 receptor agonist effective to treat drug addiction in the subject.

30

35

This invention directed to a method of treating

Alzheimer's disease in a subject which comprises administering to the subject an amount of a compound which is a GABA_BR1/R2 receptor antagonist effective to treat Alzheimer's disease in the subject.

5

This invention is directed to a peptide selected from the group consisting of:

- 10 a) P L Y S I L S A L T I L G M I M A S A F L F F N
I K N;
b) L I I L G G M L S Y A S I F L F G L D G S F V S
E K T;
c) C T V R T W I L T V G Y T T A F G A M F A K T W
R;
15 d) Q K L L V I V G G M L L I D L C I L I C W Q;
e) M T I W L G I V Y A Y K G L L M L F G C F L A
W;
f) A L N D S K Y I G M S V Y N V G I M C I I G A A
V; and
20 g) C I V A L V I I F C S T I T L C L V F V P K L I
T L R T N .

This invention is directed to a compound that prevents the formation of a GABA_BR1/R2 receptor complex.

25

Transmembrane peptides derived from GABA_BR2 sequences may modulate the functional activity of GABA_BR1/R2 receptors. One mode of action involves the destruction of the GABA_BR1/R2 receptor complex via competitive displacement of the GABA_BR2 polypeptide subunit by the peptide upon binding to the GABA_BR1 polypeptide subunit.
30 The peptides may be synthesized using standard solid phase F-moc peptide synthesis protocol using an Advanced Chemtech 396 Automated Peptide Synthesizer.

35

Additional GABA_B subtypes in hypothalamus and caudate putamen are predicted due to the under-representation of GABA_BR2 hybridization signals. These novel GABA_B

proteins and others may be identified by using GABA_BR2 polypeptides in co-immunoprecipitation experiments.

5 This invention provides a process for making a composition of matter which specifically binds to a GABA_BR1/R2 receptor which comprises identifying a chemical compound using any of the processes described herein for identifying a compound which binds to and/or activates or inhibits activation of a GABA_BR1/R2
10 receptor and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof. In one embodiment, the GABA_BR1/R2 receptor is a human GABA_BR1/R2 receptor.

15 This invention further provides a process for preparing a pharmaceutical composition which comprises admixing a pharmaceutically acceptable carrier and a pharmaceutically acceptable amount of a chemical compound identified by any of the processes described
20 herein for identifying a compound which binds to and/or activates or inhibits activation of a GABA_BR1/R2 receptor or a novel structural and functional analog or homolog thereof. In one embodiment, the GABA_BR1/R2 receptor is a human GABA_BR1/R2 receptor.

25 Thus, once the gene for a targeted receptor subtype is cloned, it is placed into a recipient cell which then expressses the targeted receptor subtype on its surface. This cell, which expresses a single population
30 of the targeted human receptor subtype, is then propagated resulting in the establishment of a cell line. This cell line, which constitutes a drug discovery system, is used in two different types of assays: binding assays and functional assays. In
35 binding assays, the affinity of a compound for both the receptor subtype that is the target of a particular drug discovery program and other receptor subtypes that

could be associated with side effects are measured. These measurements enable one to predict the potency of a compound, as well as the degree of selectivity that the compound has for the targeted receptor subtype over other receptor subtypes. The data obtained from binding assays also enable chemists to design compounds toward or away from one or more of the relevant subtypes, as appropriate, for optimal therapeutic efficacy. In functional assays, the nature of the response of the receptor subtype to the compound is determined. Data from the functional assays show whether the compound is acting to inhibit or enhance the activity of the receptor subtype, thus enabling pharmacologists to evaluate compounds rapidly at their ultimate human receptor subtypes targets permitting chemists to rationally design drugs that will be more effective and have fewer or substantially less severe side effects than existing drugs.

Approaches to designing and synthesizing receptor subtype-selective compounds are well known and include traditional medicinal chemistry and the newer technology of combinatorial chemistry, both of which are supported by computer-assisted molecular modeling. With such approaches, chemists and pharmacologists use their knowledge of the structures of the targeted receptor subtype and compounds determined to bind and/or activate or inhibit activation of the receptor subtype to design and synthesize structures that will have activity at these receptor subtypes.

Combinatorial chemistry involves automated synthesis of a variety of novel compounds by assembling them using different combinations of chemical building blocks. The use of combinatorial chemistry greatly accelerates the process of generating compounds. The resulting arrays of compounds are called libraries and are used to

screen for compounds (lead compounds) that demonstrate a sufficient level of activity at receptors of interest. Using combinatorial chemistry it is possible to synthesize focused libraries of compounds
5 anticipated to be highly biased toward the receptor target of interest.

Once lead compounds are identified, whether through the use of combinatorial chemistry or traditional medicinal
10 chemistry or otherwise, a variety of homologs and analogs are prepared to facilitate an understanding of the relationship between chemical structure and biological or functional activity. These studies define structure activity relationships which are then used to
15 design drugs with improved potency, selectivity and pharmacokinetic properties. Combinatorial chemistry is also used to rapidly generate a variety of structures for lead optimization. Traditional medicinal chemistry, which involves the synthesis of compounds one at a
20 time, is also used for further refinement and to generate compounds not accessible by automated techniques. Once such drugs are defined the production is scaled up using standard chemical manufacturing methodologies utilized throughout the pharmaceutical
25 and chemistry industry.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the
30 specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

Materials and Methods

5 DNA Sequencing

DNA sequences were determined using an ABI PRISM 377 DNA Sequencer (Perkin-Elmer, Foster City, CA) according to the manufacturer's instructions.

10 Hybridization methodology

Probes were end-labeled with polynucleotide kinase according to the manufacturer's instructions (Boehringer-Mannheim). Hybridization was performed on Zeta-Probe membrane (Bio-Rad, CA) at reduced stringency: 40°C in a solution containing 25% formamide, 5x SSC (1x SSC = 0.15 M NaCl, 0.015 M sodium citrate), 1x Denhardt's solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll, 0.02% bovine serum albumin) and 25 µg/µL sonicated salmon sperm DNA. Membrane strips were washed at 40°C in 0.1x SSC containing 0.1% SDS and exposed at -70°C to Kodak XAR film in the presence of an intensifying screen.

The nucleotide sequences of the hybridization probes are shown below:

T-891: 5'-AGGGATGCTTTCCTATGCTTCCATATTTCTCTTTGGCCTTGATGG-3' (Seq. ID No. 5) Nucleotides 1449-1493 of TL-267, forward strand.

T-892: 5'-CAATGTGCAGTTCTGCATCGTGGCTCTGGTCATCATCTTCTGCAG-3' (Seq. ID No. 6) Nucleotides 2022-2066 of TL-267, forward strand.

35 PCR Methodology

PCR reactions were carried out using a PE 9600 (Perkin-Elmer) PCR cyclers in 20 µL volumes using Expand Long

Template Polymerase (Boehringer-Mannheim) and the manufacturer's buffer 1 for internal PCR primers or manufacturer's buffer 2 for vector-anchored PCR. Reactions were run using a program consisting of 35 cycles of 94°C for 30 sec., 68°C for 20 sec, and 72°C for 1 min, with a pre-incubation at 95°C for 5 min and post-incubation hold at 4°C.

Nucleotide sequences of the primer sets used in PCR reactions are shown below:

T-94: 5'-CTTCTAGGCCTGTACGGAAGTGTT-3' (Seq. ID No. 7); vector, forward primer.

T-95: 5'-GTTGTGGTTTGTCCAAACTCATCAAT-3' (Seq. ID No. 8); vector, reverse primer.

T-887: 5'-GGGATGAGTGTCTACAACGTGGGG-3' (Seq. ID No. 9); nucleotides 1948-1971 of TL-267, forward primer.

T-888: 5'-TGCGTTGCTGCATCTGGGTTTGTCT-3' (Seq. ID No. 10); nucleotides 2138-2113 of TL-267, reverse primer.

T-889: 5'-ATCTCCCTACCTCTCTACAGCATCCT-3' (Seq. ID No. 11); nucleotides 1300-1325 of TL-267, forward primer.

T-890: 5'-CAGGTCCTGACGGTGCAAAGTGTTTC-3' (Seq. ID No. 12); nucleotides 1544-1519 of TL-267, reverse primer.

T-921: 5'-TGACGCAAGACGTTTCAGAGGTTCTCT-3' (Seq. ID No. 13); nucleotides 473-498 of TL-267, forward primer.

T-922: 5'-TGTAGCCTTCCATGGCAGCAAGCAGA-3' (Seq. ID No. 14); nucleotides 814-789 of TL-267, reverse primer.

T-923: 5'-AGAGAACCTCTGAACGTCTTGCGTCA-3' (Seq. ID No. 15); nucleotides 498-473 of TL-267, reverse primer.

T-935: 5'-GGCTCTGTTGTGTTCCACTGTAGCTG-3' (Seq. ID No. 16); nucleotides 2483-2458 of TL-267, reverse primer.

5 T-938: 5'-TCATGCCGCTCACCAAGGAGGTGGCC-3' (Seq. ID No. 17); nucleotides 53 to 78 of TL-267, forward primer.

T-939: 5'-GGCCACCTCCTTGGTGAGCGGCATGA-3' (Seq. ID No. 18); nucleotides 78 to 53 of TL-267, reverse primer.

10 T-947: 5'-TGAGTGAGCAGAGTCCAGAGCCGT-3' (Seq. ID No. 19); nucleotides -68 to -45 of TL-267, forward primer.

15 T-948: 5'-ATGGATGGGAGGTAGGCGTGGTGGAG-3' (Seq. ID No. 20); nucleotides 2591-2566 of TL-267, reverse primer.

Preparation of human hippocampal cDNA library

20 Total RNA was prepared by a modification of the guanidine thiocyanate method, from 6 grams of human hippocampus. Poly A⁺RNA was purified with a FastTrack kit (Invitrogen Corp., San Diego, CA). Double stranded (ds) cDNA was synthesized from 4 µg of poly A⁺ RNA according to Gübler and Hoffman (1983), except that ligase was omitted in the second strand cDNA synthesis. The resulting DS cDNA was ligated to BstXI/EcoRI

25 adaptors (Invitrogen Corp.), the excess of adaptors was removed by exclusion chromatography. High molecular weight fractions were ligated in pcEXV.BS (An Okayama and Berg expression vector) cut by BstXI as described by Aruffo and Seed (1987). The ligated DNA was

30 electroporated in E. coli MC 1061 (Gene Pulser, Biorad). A total of 2.2×10^6 independent clones with an insert mean size of approximately 3 kb was generated. The library was plated on Petri dishes (Ampicillin selection) in pools of 0.4 to 1.2×10^4

35 independent clones. After 18 hours amplification, the bacteria from each pool were scraped, resuspended in 4 mL of LB media and 1.5 mL processed for plasmid

purification by the alkali method (Sambrook et al, 1989). 1 mL aliquots of each bacterial pool were stored at -85°C in 20% glycerol.

5 BLAST Search that Identified a Novel 7-TM protein
 Sequence

Sequence analysis was performed with the Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, Wisconsin. The rat GABA_BR1a amino acid
10 sequence (Kaupmann et al. (1997) Nature 386:239) was used as a query to search the EST division of GenBank with BLAST. Two entries, T07621 and Z43654, had probability scores that suggested significant amino acid homology to the GABA_BR1a polypeptide. T07621 had
15 sequence homology from the beginning of the first transmembrane domain to the beginning of third transmembrane domain of the GABA_BR1a polypeptide. Z43654 had sequence homology from the sixth transmembrane domain to the seventh transmembrane
20 domain of the GABA_BR1a polypeptide. The sequence documentation for T07621 and Z43654 was retrieved with Entrez (NCBI) and neither sequence was annotated as having homology to any 7-TM spanning protein.

25 T07621 and Z43654 are part of the same sequence.

A series of PCR reactions were carried out on human hippocampus DNA with multiple primer sets: primer set T-887/T-888 designed to Z43654 sequence; primer set T-889/T-890 designed to the T07621 sequence; and primer
30 set T-889/T-888 designed to the forward strand of T07621 and the reverse stand of Z43654. The PCR products was loaded on duplicate lanes of an agarose gel and the DNA was southern blotted to a Zeta-Probe membrane (Bio-Rad, CA). The regions of the membrane
35 corresponding to the individual lanes on the gel were cut to produce membrane strips that contained duplicate samples of the DNA. One set of membrane strips was

hybridized with T-891, a probe specific for the T07621 sequence. Another set of membranes was hybridized with T-892, a probe specific to the Z43654 sequence. The membrane from primer set T-887/T-888 hybridized with probe T-892 for the Z43654 sequence. The membrane from primer set T-889/T-890 hybridized with probe T-891 for the T07621 sequence. The membrane from primer set T889/T-888 hybridized with both the T-891 and T-892 probes.

Isolating the full-length human cDNA by PCR Sib Selection.

PCR reactions were carried out on bacterial pools containing a human hippocampus cDNA library. Primer set T-888/T-889 was used to identify the bacterial pools that contained a portion of the novel receptor. Vector-anchored PCR was carried out on the positive pools to determine which pool contained the longest cDNA insert. Four primer sets were used for the vector-anchored PCR: T-94/T-888, T-94/T889, T-95/T888, and T-95/T889. Pool 365 was identified having the longest cDNA inset and the plasmid was sib selected (McCormick, 1987). The nucleotide sequence of clone 365-9-7-4, designated TL-260, was translated into amino acids and compared to the amino acid sequence of the rat GABA_BR1a polypeptide. Relative the rat GABA_BR1a amino acid sequence, TL-260 was truncated at the amino terminus.

A set of PCR primers (T-921/T-922) was made to the 5' region of TL-260 and was used to re-screen the bacterial pools of the human hippocampus library for the missing segment of the novel clone. Vector-anchored PCR was carried out on the positive pools to determine which pool contained the longest cDNA insert. Four primer sets were used for the vector-anchored PCR: T-94/T-921, T-94/T922, T-95/T921, and T-95/T-922. Pool

299 contained the most 5' sequence. A PCR product derived from the primer set T-94/T-923 was isolated (T-261) and sequenced. The putative amino acids derived from TL-261 were compared to the rat GABA_BR1 sequence. TL-261 contained an initiation codon but didn't contain a stop codon upstream of the initiation codon.

A set of PCR primers (T-938/T-935) was made to the 5' region of TL-261 and was used to re-screen the bacterial pools of the human hippocampus library for additional sequence. Vector-anchored PCR was carried out on the positive pools to determine which pool contained the longest cDNA insert. Four primer sets were used for the vector-anchored PCR: T-94/T-938, T-94/T939, T-95/T938, and T-95/T-939. A PCR product derived from primer set T-95/T-939 was isolated (T-261a) and sequenced. The putative amino acids derived from T-261a were compared to the rat GABA-1 amino acid sequence. T-261a contained an initiation codon and an in-frame upstream stop codon.

From the vector-anchored PCR, pool 389 contained the longest cDNA insert. This pool was sib selected with the primer set T-947/T-935. The resulting plasmid, 389-20-29-2, was designated TL-266 and was sequenced.

Construction of GABA_BR2 polypeptide in expression vector

A Cla-I-Xba-I fragment from TL-266 was subcloned into the expression vector pEXJ.HRT3T7 and designated TL-267. This plasmid (TL-267) was deposited on June 10, 1997, with the American Type Culture Collection (ATCC), 12301 Parklawn Drive, Rockville, Maryland 20852, U.S.A. under the provisions of the Budapest Treaty for the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure and was accorded ATCC Accession No. 209103.

Generation of rat GABA_BR2 PCR product

cDNA from rat hippocampus and rat cerebellum were amplified in 50 μ L PCR reaction mixtures using the Expand Long Template PCR System (as supplied and described by the manufacturer, Boehringer Mannheim) using a program consisting of 40 cycles of 94°C for 1 min, 50°C for 2 min, and 68°C for 2 min, with a pre- and post-incubation of 95°C for 5 min and 68°C for 7 min, respectively. PCR primers for rat GABA_BR2 were designed against the human GABA_BR2 sequence: BB 257, forward primer in the first transmembrane domain, and BB 258, reverse primer in the seventh transmembrane domain. The single 780 bp fragment from both rat hippocampus and rat cerebellum were isolated from a 1% agarose gel, purified using a GENECLAN III kit (BIO 101, Vista, CA) and sequenced using AmpliTaq DNA Polymerase, FS (Perkin Elmer). The sequence was run on an ABI PRISM 377 DNA Sequencer and analyzed using the Wisconsin Package (GCG, Genetics Computer Group, Madison, WI). This sequence was used to design PCR primers for the rat GABA_BR2 gene.

Construction and screening of a rat hypothalamic cDNA library

Poly A⁺ RNA was purified from rat hypothalamic RNA (Clontech) using a FastTrack kit (Invitrogen, Corp.). DS-cDNA was synthesized from 5 μ g of poly A⁺ RNA according to Gubler and Hoffman (1983) with minor modifications. The resulting cDNA was ligated to BstXI adaptors (Invitrogen, Corp.) And the excess adapters removed by exclusion column chromatography. High molecular weight fractions of size-selected ds-cDNA were ligated in pEXJ.T7, an Okayama and Berg expression vector modified from pcEXV (Miller and Germain, 1986) to contain BstXI, other additional restriction sites, and a T7 promoter. A total of 100,000 independent clones with a mean insert size of 3.7 kb were

generated. The library was amplified on agar plates (Ampicillin selection) in 48 primary pools. Glycerol stocks of the primary pools screened for a rat GABA_BR2 gene by PCR using BB265, a forward primer from the loop between transmembrane domains 3 and 4 from the sequence determined above and BB266, a reverse primer from the sixth transmembrane domain from the sequence determined above. The conditions for PCR were 1 min at 94°C, 4 min at 68°C for 40 cycles, with a pre- and post-incubation of 5 min at 95°C and 7 min at 68°C, respectively. To determine which pools had the largest inserts, positive pools were screened by PCR using the vector primers BB172 or BB173, and a gene-specific primer BB265 or BB266. One positive primary pool, I-47, was subdivided into 24 pools of 1000 clones, and grown in LB medium overnight. Two μ L of cultures were screened by PCR using primers BB172 and BB266. One positive subpool, I-47-4 was subdivided into 10 pools of 200 clones and plated on agar plates (ampicillin selection). Colonies were transferred to nitrocellulose membranes (Schleicher and Schuell, Keene, NH), denatured in 0.4 N NaOH, 1.5 M NaCl, renatured in 1M Tris, 1.5 M NaCl, and UV cross-linked. Filters were hybridized overnight at 40°C in a buffer containing 50 % formamide, 0.12 M Na₂HPO₄ (pH7.2), 0.25M NaCl, 7%SDS, 25 mg/L ssDNA and 10⁶ cpm/mL of a cDNA probe corresponding to transmembrane domains 1 to 7 of rat GABA_BR2, labeled with [³²P]dCTP (3000Ci/mmol, NEN) using a random prime labeling kit (Boehringer Mannheim). Filters were washed 1x 5 min then 2x 20 min at room temperature in 2x SSC, 0.1%SDS then 3x 20 min at 50° in 0.1x SSC, 0.1% SDS and exposed to Biomax MS film (Kodak) for 3 hours. Four closely clustering colonies which appeared to hybridize were re-screened individually by PCR using primers BB265 and BB266, primers BB265 and BB55, primers BB265 and BB56, and primers BB266 and BB55. The conditions for PCR were 30

sec at 94°C, 2.5 min at 68°C for 32 cycles, with a pre-
and post-incubation of 5 min at 95°C and 5 min at 68°C
respectively. One positive colony, I-47-4-2, was
amplified overnight in 10 mL TB media and processed for
5 plasmid purification using a standard alkaline lysis
miniprep procedure followed by a PEG precipitation.
This plasmid was designated BO54 and partially
sequenced using AmpliTaq DNA Polymerase, FS (Perkin
Elmer). The sequence was run on an ABI PRISM 377 DNA
10 Sequencer and analyzed using the Wisconsin Package
(GCG, Genetics Computer Group, Madison, WI). BO54 was
in the wrong orientation for expression in mammalian
cells. To obtain a clone in the correct orientation,
an *EcoRI* restriction fragment from BO54 was subcloned
15 into the vector pEXJ. Transformants were screened by
PCR using the primers BB56 and BB268 under the
following conditions: 30 sec at 94°C, 2.5 min at 68°C
for 32 cycles, with a pre- and post-incubation of 5 min
at 95°C and 3 min at 68°C respectively. One
20 transformant in the correct orientation was amplified
overnight in 100 ml TB media and processed for plasmid
purification using a standard alkaline lysis miniprep
procedure followed by a PEG precipitation. This
plasmid was designated BO55 and sequenced using
25 AmpliTaq DNA Polymerase, FS (Perkin Elmer). Plasmid
BO-55 was deposited with the ATCC on June 10, 1997, and
was accorded ATCC Accession No. 209104. The sequence
of BO-55 was determined using an ABI PRISM 377 DNA
Sequencer and analyzed using the Wisconsin Package
30 (GCG, Genetics Computer Group, Madison, WI).

Primers Used

BB257: 5'-CTCTCTGCCCTCACCATCCTCGGGAT-3' (Seq. ID No.
21)
35 BB258: 5'-GACTCCGGCTCGAATACCAGGCAGAG-3' (Seq. ID No.
22)
BB265: 5'-CCATGTTTGCAAAGACCTGGAGGGTCC-3' (Seq. ID No.

23)

BB266: 5'-GGTCACGCGTCAGGAAAGAGACAGCAG-3' (Seq. ID No.

24)

BB172: 5'-AAGCTTCTAGAGATCCCTCGACCTC-3' (Seq. ID No. 25)

5 BB173: 5'-AGGCGCAGAACTGGTAGGTATGGAA-3' (Seq. ID No. 26)

BB55: 5'-CTTCTAGGCCTGTACGGAAGTGTTA-3' (Seq. ID No. 27)

BB56: 5'-GTTGTGGTTTGTCCAACTCATCAATG-3' (Seq. ID No.

28)

BB268: 5'-CTGCTGTCTCTTTCCTGACGCGTGACC-3' (Seq. ID No.

10 29) .

Generation of DNA coding for rat GABA_B1b and GABA_B1a polypeptides

15 The gene encoding the rat GABA_BR1b polypeptide was obtained by screening the same rat hypothalamic library used for GABA_BR2 with primers based on the original publication of the clone by Kaupmann, et al., 1997. A partial clone lacking the first 55 nucleotides was identified and ligated to a PCR fragment containing the missing base pairs to obtain the full length clone. A
20 restriction fragment containing the entire coding region of GABA_BR1b was subcloned into the mammalian expression vector pEXJ.T7 and designated "BO58". A rat GABA_B1a polypeptide clone was obtained by ligating a
25 restriction fragment of the GABA_B1b clone, which contained the common region of the GABA_B1 gene, to a PCR product containing the GABA_B1a-specific 5' end.

In Situ Hybridization experiments for GABA_BR2 mRNA

30

Animals

Male Sprague-Dawley rats (Charles Rivers, Rochester, NY) were euthanized using CO₂, decapitated, and their brains immediately removed and rapidly frozen on
35 crushed dry ice. Coronal sections of brain tissue were cut at 11 μ m using a cryostat and thaw-mounted onto poly-L-lysine-coated slides and stored at -20°C until

use.

Tissue Preparation

5 Prior to hybridization, the tissues were fixed in 4%
paraformaldehyde/PBS pH 7.4 followed by two washes in
PBS (Specialty Media, Lavallette, NJ). Tissues were
then treated in 5 mM dithiothreitol, rinsed in DEPC-
treated PBS, acetylated in 0.1 M triethanolamine
10 containing 0.25% acetic anhydride, rinsed twice in 2 x
SSC, delipidated with chloroform then dehydrated
through a series of graded alcohols. All reagents were
purchased from Sigma (St. Louis, MO).

Radioactive In Situ Hybridization Histochemistry

15 Oligonucleotide probes, MJ79/80, corresponding to
nucleotides 354-398 and MJ109/110, corresponding to
nucleotides 952-991 of the rat GABA_BR2 cDNA, MJ94/95,
corresponding to nucleotides 151-193 of the human
GABA_BR1a cDNA, and MJ83/84, corresponding to nucleotides
20 34-71 of the rat GABA_BR1b cDNA were used to characterize
the distribution of each polypeptides's respective mRNA.
The oligonucleotides were synthesized using an Expedite
Nucleic Acid Synthesis System (PerSeptive Biosystems,
Framingham, MA) and purified using 12% polyacrylamide
25 gel electrophoresis. Additionally, sense and antisense
oligonucleotides corresponding to positions 1076-1120
of GABA_BR1b (1424-1468 of GABA_BR1a) were used (BB403 and
BB404).

30 The sequences of the oligonucleotides are:

For rat GABA_BR2:

Sense probe, MJ79:
5'- GCA ATA AAG TAT GGG CTG AAC CAT
35 TTG ATG GTG TTT GGA GGC GT -3' (Seq.
ID No. 36)

Antisense probe, MJ80:

Antisense probe, BB404:

5' - AAC GAC CAC CTC CTC CAG ACG TCT
TGT TCA AGG CCA AAG CCA AGG -3'
(Seq. ID No. 45)

5

Probes were 3'-end labeled with [³⁵S]dATP (1200Ci/mmol, NEN, Boston, MA) to a specific activity of 10⁹ dpm/μg using terminal deoxynucleotidyl transferase (Pharmacia, Piscataway, NJ). *In situ* hybridization was done with modification of the method described by Durkin, M, et al, 1995.

10

Nonradioactive *In Situ* Hybridization Histochemistry

15

Antisense/sense probes corresponding to nucleotides 354 - 398 of the rat GABA_BR2 cDNA, were 3'-end labeled with digoxigenin using TdT. The labeling reaction was carried out as outlined in the DIG/Genius System, (Boehringer Mannheim, Indianapolis, IN).

20

Conditions used in ISHH with digoxigenin-labeled probes are the same as described above. The sections were rinsed in buffer 1, washing buffer (0.1 M Tris-HCl pH 7.5/0.15 M NaCl), pre-incubated in Blocking Solution (Buffer 1 , 0.1% Triton-X and 2% normal sheep serum) for 30 minutes and then incubated for 2 hours in

25

Blocking Solution containing anti-digoxigenin-AP Fab fragment (Boehringer Mannheim) at 1:500 dilution followed by two 10 minute washes in Buffer 1. To develop color, sections were rinsed in Detection Buffer (0.1M Tris-HCl pH 9.5/0.15M NaCl/0.05 M MgCl₂) for 10 minutes and then incubated overnight in Detection Buffer containing 0.5 mM NBT, 0.1 mM BCIP, and 1 mM levamisole. After color development, slides were dipped in dH₂O and coverslipped using aqua mount.

30

35

Probe specificity was established by performing *in situ* hybridization on HEK293 cells transiently transfected with eukaryotic expression vectors containing the rat

GABA_BR1b and human GABA_BR1a DNA or no insert for transfection. Furthermore, two pairs of hybridization probes, sense and antisense, that were targeted to different segments of the GABA_BR2 mRNA were used for cells and rat tissues.

Quantification

The strength of the hybridization signal obtained in various region of the rat brain was graded as weak (+), moderate (++) , heavy (+++) or intense (++++). These were qualitative evaluations for each of the polypeptide mRNA distributions based on the relative optical density on the autoradiographic film and on the relative number of silver grains observed over individual cells at the microscopic level.

Cell Culture

COS-7 cells are grown on 150 mm plates in DMEM with supplements (Dulbecco's Modified Eagle Medium with 10% bovine calf serum, 4 mM glutamine, 100 units/mL penicillin/100 µg/mL streptomycin) at 37°C, 5% CO₂. Stock plates of COS-7 cells are trypsinized and split 1:6 every 3-4 days.

Human embryonic kidney 293 cells are grown on 150 mm plates in DMEM with supplements (10% bovine calf serum, 4 mM glutamine, 100 units/mL penicillin/100µg/mL streptomycin) at 37°C, 5% CO₂. Stock plates of 293 cells are trypsinized and split 1:6 every 3-4 days.

Mouse fibroblast LM(tk-) cells are grown on 150 mm plates in D-MEM with supplements (Dulbecco's Modified Eagle Medium with 10% bovine calf serum, 4 mM glutamine, 100 units/mL penicillin/100 µg/mL streptomycin) at 37°C, 5% CO₂. Stock plates of LM(tk-) cells are trypsinized and split 1:10 every 3-4 days.

Chinese hamster ovary (CHO) cells are grown on 150 mm plates in HAM's F-12 medium with supplements (10% bovine calf serum, 4 mM L-glutamine and 100 units/mL penicillin/100 ug/mL streptomycin) at 37°C, 5% CO₂.
5 Stock plates of CHO cells are trypsinized and split 1:8 every 3-4 days.

Mouse embryonic fibroblast NIH-3T3 cells are grown on 150 mm plates in Dulbecco's Modified Eagle Medium (DMEM)
10 with supplements (10% bovine calf serum, 4 mM glutamine, 100 units/mL penicillin/100 µg/mL streptomycin) at 37°C, 5% CO₂. Stock plates of NIH-3T3 cells are trypsinized and split 1:15 every 3-4 days.

Sf9 and Sf21 cells are grown in monolayers on 150 mm tissue culture dishes in TMN-FH media supplemented with 10% fetal calf serum, at 27°C, no CO₂. High Five insect cells are grown on 150 mm tissue culture dishes in Ex-Cell 400™ medium supplemented with L-Glutamine, also at
15 27°C, no CO₂.
20

LM(tk-) cells stably transfected with the DNA encoding the polypeptides disclosed herein may be routinely converted from an adherent monolayer to a viable
25 suspension. Adherent cells are harvested with trypsin at the point of confluence, resuspended in a minimal volume of complete DMEM for a cell count, and further diluted to a concentration of 10⁶ cells/mL in suspension media (10% bovine calf serum, 10% 10X Medium 199
30 (Gibco), 9 mM NaHCO₃, 25 mM glucose, 2 mM L-glutamine, 100 units/mL penicillin/100 µg/mL streptomycin, and 0.05% methyl cellulose). Cell suspensions are maintained in a shaking incubator at 37°C, 5% CO₂ for 24 hours. Membranes harvested from cells grown in this
35 manner may be stored as large, uniform batches in liquid nitrogen.

Alternatively, cells may be returned to adherent cell culture in complete DMEM by distribution into 96-well microtiter plates coated with poly-D-lysine (0.01 mg/mL) followed by incubation at 37°C, 5% CO₂ for 24 hours.

Generation of baculovirus

The coding region of DNA encoding the polypeptides disclosed herein may be subcloned into pBlueBacIII into existing restriction sites, or sites engineered into sequences 5' and 3' to the coding region of the polypeptides. To generate baculovirus, 0.5 µg of viral DNA (BaculoGold) and 3 µg of DNA construct encoding a polypeptide may be co-transfected into 2 x 10⁶ *Spodoptera frugiperda* insect Sf9 cells by the calcium phosphate co-precipitation method, as outlined in by Pharmingen (in "Baculovirus Expression Vector System: Procedures and Methods Manual"). The cells then are incubated for 5 days at 27°C.

The supernatant of the co-transfection plate may be collected by centrifugation and the recombinant virus plaque purified. The procedure to infect cells with virus, to prepare stocks of virus and to titer the virus stocks are as described in Pharmingen's manual.

Transfection

All subtypes studied may be transiently transfected into COS-7 cells by the DEAE-dextran method, using 1 µg of DNA /10⁶ cells (Cullen, 1987). In addition, Schneider 2 *Drosophila* cells may be cotransfected with vectors containing the gene, under control of a promoter which is active in insect cells, and a selectable resistance gene, eg., the G418 resistant neomycin gene, for expression of the polypeptides disclosed herein.

Stable Transfection

DNA encoding the polypeptides disclosed herein may be co-transfected with a G-418 resistant gene into the human embryonic kidney 293 cell line by a calcium phosphate transfection method (Cullen, 1987). Stably transfected cells are selected with G-418.

Radioligand binding assays

Transfected cells from culture flasks were scraped into 5 mL of Tris-HCl, 5mM EDTA, pH 7.5, and lysed by sonication. The cell lysates were centrifuged at 1000 rpm for 5 min. at 4°C, and the supernatant was centrifuged at 30,000 x g for 20 min. at 4°C. The pellet was suspended in binding buffer (50 mM Tris-HCl, 2.5 mM CaCl₂ at pH 7.5 supplemented with 0.1% BSA, 2µg/mL aprotinin, 0.5mg/mL leupeptin, and 10µg/mL phosphoramidon). Optimal membrane suspension dilutions, defined as the protein concentration required to bind less than 10% of the added labeled compound (typically a radiolabeled compound), were added to 96-well polypropylene microtiter plates containing labeled compound, unlabeled compounds (i.e., displacing ligand in an equilibrium competition binding assay) and binding buffer to a final volume of 250 µL. In equilibrium saturation binding assays membrane preparations were incubated in the presence of increasing concentrations of labeled compound. The binding affinities of the different compounds were determined in equilibrium competition binding assays, using labeled compound, such as 1 nM [³H]-CGP54626, in the presence of ten to twelve different concentrations of the displacing ligand(s). Some examples of displacing ligands included GABA, baclofen, 3APMPA, phaclofen, CGP54626, and CGP55845. Mixtures of several unlabeled test compounds (up to about 10 compounds) may also be used in competition binding assays, to determine whether one of the mixture component

compounds binds to the polypeptide or receptor. Binding reaction mixtures were incubated for 1 hr at 30°C, and the reaction was stopped by filtration through GF/B filters treated with 0.5% polyethyleneimine, using a cell harvester. Where the labeled compound was a radiolabeled compound, the amount of bound compound was evaluated by gamma counting (for ^{125}I) or scintillation counting (for ^3H). Data were analyzed by a computerized non-linear regression program. Non-specific binding was defined as the amount of radioactivity remaining after incubation of membrane protein in the presence of excess unlabeled compound. Protein concentration may be measured by the Bradford method using Bio-Rad Reagent, with bovine serum albumin as a standard.

Cyclic AMP (cAMP) formation assay

The receptor-mediated inhibition of cyclic AMP (cAMP) formation may be assayed in transfected cells expressing the mammalian receptors described herein. Cells are plated in 96-well plates and incubated in Dulbecco's phosphate buffered saline (PBS) supplemented with 10 mM HEPES, 5mM theophylline, 2 $\mu\text{g/ml}$ aprotinin, 0.5 mg/ml leupeptin, and 10 $\mu\text{g/ml}$ phosphoramidon for 20 min at 37°C, in 5% CO_2 . Test compounds are added and incubated for an additional 10 min at 37°C. The medium is then aspirated and the reaction stopped by the addition of 100 mM HCl. The plates are stored at 4°C for 15 min, and the cAMP content in the stopping solution measured by radioimmunoassay. Radioactivity may be quantified using a gamma counter equipped with data reduction software.

Generation of chimeric G-proteins

Chimeric G-proteins were constructed using standard mutagenesis methods(Conklin *et al.*, 1993). Two chimeras were constructed. The first comprises the

entire coding region of human G_{aq} with the exception of the final 3' 15 nucleotides which encode the C-terminal 5 amino acids of G_{i3} . The second also comprises the entire coding region of human G_{aq} with the exception of the final 3' 15 nucleotides which encode the C-terminal 5 amino acids of G_{i2} . Sequences of both chimeric G-protein genes were verified by nucleotide sequencing. For the purposes of expression in oocytes, synthetic mRNA transcripts of each gene were synthesized using the T7 polymerase.

Phosphoinositide Assay

The agonist activities of GABA-B agonists were assayed by measuring their ability to generate phosphoinositide production in COS-7 cells transfected transiently with $GABA_B R1$, $GABA_B R2$, and chimeric $G_{aq/i2}$. Alternatively, COS-7 cells are transfected transiently with $GABA_B R1$, $GABA_B R2$, and other chimeric G-protein alpha subunits such as $G_{aq/i2}$, $G_{aq/i3}$, or $G_{aq/o}$. Cells were plated in 96-well plates and grown to confluence. The day before the assay the growth medium was changed to 100 ml of medium containing 1% serum and 0.5 mCi [3H]myo-inositol, and the plates were incubated overnight in a CO_2 incubator (5% CO_2 at 37°C).

Immediately before the assay, the medium was removed and replaced by 200 ml of PBS containing 10 mM LiCl, and the cells were equilibrated with the new medium for 20 min. The [3H]inositol-phosphate (IP) accumulation was started by adding 22 ml of a solution containing the agonist. To the first two wells 22 ml of PBS were added to measure basal accumulation, and 10 different concentrations of agonist were assayed in the following 10 wells of each plate row. All assays were performed in duplicate by repeating the same additions in two consecutive rows. The plates were incubated in a CO_2 incubator for 30 min. The reaction was terminated by

removal of the buffer solution by blotting, followed by the addition of 100 μ l of 50% (v/v) trichloroacetic acid (TCA), and 10 min incubation at 4°C.

The contents of the wells were then transferred to a Multiscreen HV filter plate (Millipore) containing Dowex AG1-X8 (200-400 mesh, formate form). The filter plates were prepared adding 100 ml of Dowex AG1-X8 suspension (50% v/v, water:resin) to each well. The filter plates were placed on a vacuum manifold to wash or elute the resin bed. Each well was washed 3 times with 200 μ l of 5mM myo-inositol. The [3 H]-IPs were eluted into empty 96-well plates with 75 ml of 1.2 M ammonium formate/0.1 M formic acid. After the addition of 200 μ l of scintillation cocktail (Optiphase Supermix; Wallac) to each well, [3 H]-IPs were quantified by counting on a Trilux 1450 Microbeta scintillation counter.

Oocyte expression

Female *Xenopus laevis* (Xenopus-1, Ann Arbor, MI) are anesthetized in 0.2% tricain (3-aminobenzoic acid ethyl ester, Sigma Chemical Corp.) and a portion of ovary is removed using aseptic technique (Quick and Lester, 1994). Oocytes are defolliculated using 3 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ) in a solution containing 87.5 mM NaCl, 2 mM KCl, 2 mM MgCl₂ and 5 mM HEPES, pH 7.5. Oocytes are injected (Nanoject, Drummond Scientific, Broomall, PA) with 50-70 nl mRNA prepared as described below. After injection of mRNA, oocytes are incubated at 17 degrees for 3-8 days.

RNAs are prepared by transcription from: (1), linearized DNA plasmids containing the complete coding region of the gene, or (2), templates generated by PCR

incorporating a T7 promoter and a poly A⁺ tail. From either source, DNA is transcribed into mRNA using the T7 polymerase ("Message Machine", Ambion).

The transcription template for the rat GABA_BR1b gene was prepared by PCR amplification of the plasmid BO58 using the primers MJ23 and MJ47 (see below). The template for the rat GABA_BR2 gene was made by linearization of the plasmid BO56, rat GABA_BR2 insert from BO55 in the expression vector pEXJ.T7, with NotI.

Primers:

MJ23 5'
CCAAGCTTCTAATACGACTCACTATAGGGGAGACCATGGGCCCGGGGG
ACCCTGTACC 3' (Seq. ID No. 30);

MJ47 5' T₍₃₅₎CACTTGTAAGCAAATGTACTCGACTCC 3' (Seq. ID No. 31).

Genes encoding G-protein inwardly rectifying K⁺ channels 1 and 4 (GIRK1 and GIRK4; "GIRKs") were obtained by PCR using the published sequences (Kubo et al., 1993; Dascal et al., 1993; Krapivinsky et al., 1995b) to derive appropriate 5' and 3' primers. Human heart cDNA was used as template together with the primers

5'-CGCGGATCCATTATGTCTGCACTCCGAAGGAAATTTG-3' (Seq. ID No. 32) and

5'-CGCGAATTCTTATGTGAAGCGATCAGAGTTCATTTTTC -3' (Seq. ID No. 33) for GIRK1 and

5'-GCGGGATCCGCTATGGCTGGTGATTCTAGGAATG-3' (Seq. ID No. 34) and

5'- CCGGAATTCCCCTCACACCGAGCCCCTGG-3' (Seq. ID No. 35) for GIRK4.

The BamH1 and EcoR1 restriction sites in each primer

pair were used to clone the PCR product into the expression vector pcDNA-Amp (Invitrogen). Plasmid vectors containing GIRK1 and GIRK4 are referred to as "JS1800" and "JS1741", respectively. The coding regions of both genes were sequenced and verified.

Oocyte electrophysiology

Dual electrode voltage clamp ("GeneClamp", Axon Instruments Inc., Foster City, CA) is performed using 3 M KCl-filled glass microelectrodes having resistances of 1-3 Mohms. Unless otherwise specified, oocytes are voltage clamped at a holding potential of -80 mV. During recordings, oocytes are bathed in continuously flowing (1-3 ml/min) medium containing 96 mM NaCl, 2 mM KCl, 1.8 mM CaCl_2 , 1 mM MgCl_2 , and 5 mM HEPES, pH 7.5 (ND96), or elevated K^+ containing 49 mM KCl, 49 mM NaCl, 1.8 mM CaCl_2 , 2 mM MgCl_2 , and 5 mM HEPES, pH 7.5 (hK). Drugs are applied either by local perfusion from a 10 μl glass capillary tube fixed at a distance of 0.5 mm from the oocyte, or for calculation of steady-state EC_{50}s , by switching from a series of gravity fed perfusion lines. Experiments are carried out at room temperature. All values are expressed as mean \pm standard error of the mean.

Concentration-response curves for agonists and antagonists were fitted with logistic equations of the form $I = 1/(1 + (\text{EC}_{50}/[\text{Agonist}])^n)$ for agonists and $I = 1/(1 + ([\text{Antagonist}]/\text{IC}_{50})^n)$ for antagonists, where I is current, where EC_{50} is the concentration of agonist that produced half-maximal activation, IC_{50} is the concentration of antagonist that produced half-maximal inhibition, and n the Hill coefficient. Fits were made with a Marquardt-Levenberg non-linear least-squares curve fitting algorithm.

Recording ion currents in mammalian cells

The ability of the rat GABA_BR1 and GABA_BR2 genes to activate GIRK currents in mammalian cells was investigated by transient transfection of HEK-293 cells followed by voltage clamp analysis of currents. HEK-293 cells were maintained in Dulbecco's modified Eagle medium (DMEM) plus 10% (v/v) bovine calf serum, 2% L-glutamine, 50 U/ml penicillin, and 50 µg/ml streptomycin and were incubated at 37° C in a humidified 5% CO₂ atmosphere. Cells were harvested twice each week by treatment with 0.25% trypsin/1 mM EDTA in Hank's Salts and re-seeded at 20% of their original density either into 75 cm² flasks (for passaging) or into 35 mm tissue culture dishes (for transfection and electrophysiology experiments).

HEK-293 cells, 40% - 80% confluent, were co-transfected with various combinations of 0.6 µg each of the following plasmids: pGreen Lantern-1 (Gibco/BRL, Gaithersburg, MD), human GIRK1 (JS1800), human GIRK4 (JS1741), rat GABA_BR1b (BO58), and rat GABA_BR2 (BO55). Cells were transiently transfected using the Superfect Transfection Reagent from Qiagen (Valencia, CA) according to the manufacturer's instructions. Briefly, 3 µg total plasmid DNA were incubated with 22.5 µl Superfect Reagent in 100 µl serum-free DMEM for 5-10 minutes at room temperature. After addition of 600 µl complete DMEM, the DNA/Superfect mixture was transferred to cells growing in 35 mm dishes coated with poly-D-lysine and incubated for 2-4 hours at 37° C in a 5% CO₂ incubator. Subsequently, the dishes were washed once with phosphate-buffered saline and 2 ml complete DMEM was added. Cells were incubated for 24-72 hours at 37° C before performing electrophysiological measurements.

The whole-cell configuration of the patch-clamp technique was used with glass pipettes having resistances of 2-4 MΩ when filled with the pipette

0931156 12153
866727 9971660

solution. Solutions used were (in mM), KMeSO₄, 125; KCl, 5; NaCl, 5; MgCl₂, 2; EGTA, 11; HEPES, 10, pH 7.4; MgATP, 1.0; Na₂GTP, 0.2, for the pipette and NaCl, 130; KCl, 4; CaCl₂, 2; MgCl₂, 2; Glucose, 10; Sucrose, 10; HEPES, 10, pH 7.4 for the bath. GIRK currents were recorded in elevated K⁺ solution containing 25 mM K⁺ and a correspondingly lower concentration of Na⁺. Voltage clamp recordings were made with an EPC-9 amplifier using Pulse+PulseFit software (HEKA Elektronik). Series resistances were kept below 10 Mohm and no attempt was made to provide series resistance compensation. Currents were low-pass filtered at 1 kHz and digitized at a rate of 5 kHz. Unless otherwise noted, experiments were performed at room temperature on cells voltage clamped at a holding potential of -70 mV. Application of agonists was realized using a gravity-fed, perfusion system consisting of six concentrically arranged microcapillary tubes (Jones et al. 1997). The time to complete solution exchange was about 100 ms. The bath was constantly perfused at a low rate with control solution.

All voltage clamp recordings were made from transfected cells visualized under epifluorescent lighting conditions utilizing a filter set designed for GFP (Zeiss Optics). Fluorescent cells were an excellent indication of transfection since they all exhibited some constitutive GIRK current activity in contrast to untransfected cells which displayed no measurable inward rectifier K⁺ currents (data not shown).

Microphysiology

GABA_BR1, GABA_BR2 or the combination, were transiently expressed in CHO-K1 cells by liposome mediated transfection according to the manufacturer's recommendations ("LipofectAMINE", GibcoBRL, Bethesda,

MD), and maintained in Ham's F-12 medium with 10% bovine serum. Cells were prepared for microphysiometric recording as previously described (Salon, J. A., et al., 1995). On the day of the experiment the cell capsules were transferred to the microphysiometer and allowed to equilibrate in recording media (low buffer RPMI 1640, no bicarbonate, no serum, Molecular Devices Corp.), during which a baseline was established. The recording paradigm consisted of a 100 ml/min flow rate and a 30 s flow interruption during which the rate measurement was taken. Challenges involved an 80 s drug exposure just prior to the first post-challenge rate measurement being taken, followed by two additional pump cycles. Acidification rates reported are expressed as a percentage increase of the peak response over the baseline rate observed just prior to challenge.

N-terminal deletion experiments

As a start to exploring the structural aspects of GABA_BR2 important for functional activity of the GABA_BR1/R2 receptor, N-terminal deletion experiments were performed on the GABA_BR2-HA construct (see below). All such deletion mutants caused a complete disruption of receptor activity as assessed by the measurement of GIRK currents in transfected HEK293 cells. In one such experiment, wildtype GABA_BR2-HA was digested with BglII restriction enzyme and religated. The BglII deletion mutant (M118) lacks 257 amino acids at the N-terminus, corresponding to positions 226-482. Using immunofluorescence, M118 was found to be expressed on the cell surface, similarly to the wildtype GABA_BR2-HA, yet when co-expressed with GABA_BR1 did not produce GIRK activation with 100 μ M GABA. Thus, although we cannot yet identify specific amino acids contributing to receptor activity, it appears that the N-terminal

region comprising amino acids 226-482 is critically important either for dimer formation, ligand binding or conformational changes associated with signal transduction.

5

Construction of epitope-tagged polypeptides and confocal microscopy

10 Incorporation of sequences encoding the RGS6xHis or influenza virus hemagglutinin (HA) epitope into the GABA_BR1 and GABA_BR2 genes, respectively, was performed by PCR. Each epitope was positioned immediately before the stop codon in the appropriate gene. Both tagged genes were subcloned into pCDNA. Sequence analysis was used to confirm all PCR-derived portions of the
15 construct. Forty-eight hours post-transfection HEK293 cells were fixed for 20 min in 4% paraformaldehyde in PBS, permeablized in PBS containing 2% BSA and 0.1% Triton X-100 and incubated with primary antibody for 1.5 h. Mouse monoclonal anti-RGS (Qiagen) and mouse anti-FLAG (Boehringer-Mannheim) were labeled with FITC-
20 conjugated goat anti-mouse antibodies. Rat monoclonal anti-HA (Boehringer-Mannheim) was visualized with TRITC-conjugated rabbit anti-irat antibodies. Fluorescent images were obtained with a Zeiss LSM 410
25 confocal microscope using a 100x oil-immersion objective.

Immunoprecipitation and Western blotting

30 Forty-eight hours following transient transfection HEK293 cells were solubilized in lysis buffer containing (in mM): 50 Tris/Cl pH 7.4, 300 NaCl, 1.5 MgCl₂, 1 CaCl₂, protease inhibitors (Boehringer Mannheim tablets), 1% Triton X-100, and 10% glycerol. 1-2 mg of

protein was immunoprecipitated overnight at 4° C with
either 0.5 µg rat monoclonal anti-HA antibody or 0.5 µg
mouse monoclonal anti-4xHis antibody (Qiagen). Immune
complexes were bound to 20 µl Protein-A agarose
5 (Research Diagnostics, Inc.) for 2 h at RT. Protein-A
pellets were washed twice with buffer containing
Triton-X-100, then once without, and eluted with 80 µl
Laemmli sample buffer containing 2% (w/v) SDS and 20 mM
DTT. After heating for 3 min. at 70° C, 20 µl IP samples
10 or 20 µg total protein was subjected to SDS-PAGE
followed by Western blotting with either anti-HA or
anti-4xHis antibody, followed by sheep anti-rat
(Amersham) or goat anti-mouse (RDI) HRP-linked
secondary antibodies, respectively. Proteins were
15 visualized with enhanced chemiluminescent substrates
(Pierce).

Alternatively, material for immunoprecipitations was
obtained by sucrose gradient fractionation of the P1
pellet as described by Graham(Graham, 1984). To verify
20 the enrichment of plasma membrane in the resulting
"P1+" pellet, Na⁺/K⁺ ATPase in the P1+ and P2 (primarily
microsomal and vesicular(Graham, 1984)) fractions was
quantified by fluorescence detection of anti-alpha 1
subunit antibody (Research Diagnostics, Inc., clone 9A-
25 5) on a phosphor imager (Molecular Dynamics). ATPase
in P1+ fractions used for immunoprecipitations was
found to be enriched >50 fold compared to P2 fractions.

Experimental Results

Novel GPCR sequences identified by BLAST search

The rat GABA_BR1a amino acid sequence (Kaupmann *et al.* (1997) Nature 386:239) was used as a query to search the EST division of GenBank with BLAST. Two entries, T07621 and Z43654, had probability scores that suggested significant amino acid homology to the GABA_BR1a polypeptide. T07621 had sequence homology from the beginning of the first transmembrane domain to the beginning of third transmembrane domain of the GABA_BR1a polypeptide. Z43654 had sequence homology from the sixth transmembrane domain to the seventh transmembrane domain of the GABA_BR1a polypeptide. The sequence documentation for T07621 and Z43654 was retrieved with Entrez (NCBI) and neither sequence was annotated as having homology to any 7-TM spanning protein.

These results were used to obtain a full-length human clone TL-266, comprising both of the sequences identified by the BLAST search. Sequence analysis of clone TL-266 revealed a complete coding region for a novel protein. A search of the GenEMBL database indicated that the most similar sequence was that of GABA_BR1a, followed by G protein-coupled receptors (GPCRs) of the metabotropic receptor superfamily. The nucleotide and deduced amino acid sequence of TL-267 are shown in Figures 1 and 2, respectively. The nucleotide sequence of the coding region is 57% identical to the rat GABA_BR1a over a region of 1,686 bases. The longest open reading frame encodes an 898 amino acid protein with 38% amino acid identity to the rat GABA_BR1a polypeptide. Hydropathy plots of the predicted amino acid sequence reveal seven hydrophobic regions that may represent transmembrane domains (TMs, data not shown), typical of the G protein-coupled

receptor superfamily. In the putative TM domains, GABA_BR2 exhibits 45% amino acid identity with the rat GABA_BR1a polypeptide. The amino terminus of TL-266 has amino acid homology to the bacterial periplasmic binding protein, a common feature of the metabotropic receptors (O'Hara et al. (1993) Neuron 11:41-52).

Generation of rat GABA_BR2 PCR Product

Using PCR primers designed against the first and seventh transmembrane domains of the human GABA_BR2 sequence, BB257 and BB258, a 780 base pair fragment was amplified from rat hippocampus and rat cerebellum. Sequence from these bands displayed 90% nucleotide identity to the human GABA_BR2 gene. This level of homology is typical of a species homologue relationship in the GPCR superfamily.

Construction and Screening of a Rat Hypothalamic cDNA Library

To obtain a full-length rat GABA_BR2 clone, pools of a rat hypothalamic cDNA library were screened by PCR using primers BB265 and BB266. A 440 base pair fragment was amplified from 44 out of 47 pools. Vector-anchored PCR was performed to identify pools with the largest insert size. One positive primary pool, I-47, was subdivided into 24 pools of 1000 individual clones and screened by vector-anchored PCR. Seven positive subpools were identified and one, I-47-4, was subdivided into 10 pools of 200 clones, plated onto agar plates, and screened by southern analysis. Four closely clustering colonies that appeared positive were rescreened individually by vector-anchored PCR. One positive colony, I-47-4-2, designated B054, was amplified as a single rat GABA_BR2 clone. Since vector-anchored PCR revealed that B054 was in the wrong

orientation for expression, the insert was isolated by restriction digest and subcloned into the expression vector pEXJ. A transformant in the correct orientation was identified by vector-anchored PCR, and designated BO-55.

The nucleotide and deduced amino acid sequence of BO-55 are shown in Figures 3 and 4, respectively. BO-55 contains a 2.82 kB open reading frame and encodes a polypeptide of 940 amino acids. The nucleotide sequence of BO-55 is 89% identical to TL-267 in the coding region, with an overall amino acid identity of 98%. The proposed signal peptide cleavage site is between amino acids 40 and 41 (Nielsen et al., 1997).

A BLAST search of GenEMBL indicated that this sequence was most closely related to GABA_BR1, displaying 35% and 41% amino acid identities overall and within the predicted transmembrane domains, respectively (Fig. 10). The structural similarity to GABA_BR1 indicated that this sequence encodes a novel polypeptide, which we refer to as GABA_BR2. The next most related sequences were other members of the mGluR family, with 21-24% overall amino acid identities. Like GABA_BR1 and other members of the mGluR family (O'Hara, P. J., et al., 1998), GABA_BR2 contains a large N-terminal extracellular domain having regions of homology to bacterial periplasmic binding proteins.

Distribution of GABA_BR1 or GABA_BR2 in cDNA libraries

Three cDNA libraries were screened by PCR with primers directed to transmembrane regions of either GABA_BR1 or GABA_BR2. In a human hippocampal cDNA library both polypeptides were found in greater than 90% of the pools and in a rat hypothalamic cDNA library, again both polypeptides were found in greater than 90% of the

5 pools. In addition, within each of these two
libraries, the respective frequency of GABA_BR1 and
GABA_BR2 seems to be the same. However, in a rat spinal
cord cDNA library, GABA_BR1 was found in 62.5% of the
pools while GABA_BR2 was found in only 17.5% of the
pools. Thus, while both polypeptide subtype appear to
be present at high frequency in all three of the
libraries, in the spinal cord library GABA_BR2 occurs at
3.6-fold lower frequency. These data point to the
10 existence of an additional GABA_B-like peptide(s).

Results of Localization

Controls

15 The specificity of the hybridization of the GABA_BR2
probe was verified by performing *in situ* hybridization
on transiently transfected HEK293 cells as described in
Methods. The results indicate that hybridization to
each of the individual GABA_B polypeptides was specific
only to the HEK293 cells transfected with each
20 respective cDNA.

In addition, *in situ* hybridization on rat brain
sections was performed using two hybridization probes
targeted to different segments of the GABA_BR2 mRNA. In
each case the pattern and intensity of labeling was
25 identical in all regions of the rat CNS. Nonspecific
hybridization signal was determined using the sense
probes and was indistinguishable from background.

Localization of GABA_BR2 mRNA in rat CNS

30 The anatomical distribution of GABA_BR2 mRNA in the rat
brain was determined by *in situ* hybridization. By
light microscopy the silver grains were determined to
be distributed over neuronal profiles. The results
suggest that the mRNA for GABA_BR2 is widely distributed

throughout the rat CNS in addition to several sensory ganglia (Figures 19H-I). However, expression levels in the brain were not uniform with several regions exhibiting higher levels of expression such as the medial habenula, CA3 region of the hippocampus, piriform cortex, and cerebellar Purkinje cells (Figures 19A-F). Moderate expression levels were observed in the ventral pallidum, septum, thalamus, CA1 region of the hippocampus, and geniculate nuclei (Figures 19C,D,E). Lower expression of GABA_BR2 mRNA was seen in the hypothalamus, mesencephalon, and several brainstem nuclei (Figures 19D,F). GABAergic neurons and terminals are likewise widely distributed in the CNS (Mugnaini, E., et al., 1985). and the distribution of the GABA_BR2 mRNA correlates well with the distribution of GABAergic neurons. One exception is the substantia nigra which contains high densities of GABAergic neurons, yet very low expression of GABA_BR2 mRNA. Additionally, the anatomical distribution of GABA_BR2 mRNA is in concordance with previous reports of the distribution of GABA_B binding sites obtained using [³H]baclofen (Gehlert, D. R., et al., 1985), and [³H]GABA (Bowery, N. J., et al., 1987). Furthermore, there was a high degree of similarity in the distribution and intensity of GABA_BR2 hybridization signal relative to those previously reported for GABA_BR1 (Bischoff, S., et al., 1997) (Figures 11, 12). Notable exceptions were the hypothalamus and caudate-putamen, where the expression of GABA_BR2 message appeared lower than that of GABA_BR1.

Colocalization of GABA_BR2 and GABA_BR1b mRNAs in the rat CNS

The results of the *in situ* hybridization studies using digoxigenin-labeled probe conjugated to alkaline phosphatase and the chromagen NBT/BCIP for the GABA_BR2

mRNA and an [³⁵S]dATP-labeled probe for the GABA_BR1b mRNA indicated that coexpression of the GABA_BR2 mRNA and GABA_BR1b mRNA did occur *in vivo* in neurons. In particular, colocalization was observed in cells of the medial habenula, hippocampus, and the cerebellar Purkinje cells. Likewise, there was evidence from the autoradiograms for potential overlapping distribution of the three known GABA_B mRNAs in the olfactory bulb, throughout the entire neocortex, several hypothalamic nuclei, numerous thalamic nuclei and brain stem nuclei. However, the Purkinje cells of the cerebellum contained message for only GABA_BR2 and GABA_BR1b and not the GABA_BR1a. Additionally, all three subtypes appear to be distributed throughout the gray matter of the spinal cord in all levels of the spinal cord.

The overlapping expression patterns of GABA_BR1 and GABA_BR2 transcripts in the brain suggested the possibility the polypeptides may be co-expressed in individual neurons and that both might be required for functional activity.

Oocyte expression

Postsynaptic inhibition of neurons by GABA_B receptor activation is caused by the opening of inwardly rectifying K⁺ channels (GIRK) (North, R. A., 1989; Andrade, R. et al., 1986; Gahwiler, B. H., et al., 1985; Luscher, C., et al., 1997). Oocytes expressing the combination of GABA_BR1b and GABA_BR2 mRNAs together with GIRKs elicited large currents in response to 30 μM GABA (Table 1a and 1b). (Subsequent to the compilation of the data in Table 1a, experiments were done to make Table 1b.) GABA and baclofen evoked sustained currents of similar magnitude (Fig. 13B). In contrast, oocytes expressing transcripts encoding either GABA_BR1a, GABA_BR1b, or GABA_BR2 alone consistently failed to generate GIRK currents in response to high

concentrations of GABA (1 mM), baclofen (1 mM) or 3-APMPA (100 μ M). Others have reported similar results with GABA_AR1 (Kaupmann, K. et al., 1997a; Kaupmann, K., et al., 1997b).

Table 1a. Magnitude of GIRK currents stimulated by GABA in oocytes and HEK-293 cells expressing GIRK1 and GIRK4 and various combinations of rat GABA_BR1 and rat GABA_BR2.

	Oocytes			mean	HEK-293	
	mean	S.E.M.	(n)		S.E.M.	(n*)
	(nA)			(pA)		
GABA _B R1a	0	0	(35)	-	-	-
GABA _B R1b	0	0	(15)	5	3	(3/26)
GABA _B R2	0	0	(19)	5	5	(1/6)
GABA _B R1b	1396	269	(7)	658	323	(9/10)
+ GABA _B R2						
GABA _B R1b	7	7	(2)	-	-	-
+ GABA _B R2						
+ PTX						

* number of cells responding / total number studied

Table 1b. Magnitude of GIRK currents stimulated by GABA in oocytes and HEK-293 cells expressing GIRK1 and GIRK4 and various combinations of rat GABA_BR1 and rat GABA_BR2.

	Oocytes			mean	HEK-293	
	mean	S.E.M.	(n)		S.E.M.	(n*)
	(nA)			(pA)		
GABA _B R1a	0	0	(35)	-	-	-
GABA _B R1b	0	0	(23)	5	3	(5/26)
GABA _B R2	0.230	.13	(30)	.87	.87	(1/23)
GABA _B R1b	832	65	(65)	470	71	(70/81)
+ GABA _B R2						
GABA _B R1b	16	9	(3)	-	-	-
+ GABA _B R2						
+ PTX						

* number of cells responding / total number studied

Currents stimulated by GABA in oocytes injected with both GABA_R1b and GABA_R2 mRNAs were completely blocked by the selective antagonist CGP55845 (1 μ M) in a reversible fashion (data not shown). The potency of GABA and baclofen for eliciting GIRK currents was measured by performing steady-state cumulative concentration response assays on individual oocytes (Figure 6A). Like K⁺ responses elicited by stimulation of native GABA_R receptors (Lacy et al. 1988; Misgeld et al. 1995), responses in oocytes did not desensitize and could be faithfully reproduced by multiple agonist applications on single oocytes. Stimulation of inward current was concentration dependent for both GABA and baclofen. The EC₅₀s, 1.76 μ M for GABA and 3.99 μ M for baclofen (Figure 6B, Figure 7), agreed closely with those reported in the literature for native receptors (Lacy et al. 1988; Misgeld et al. 1995). Concentration-effect curves for GABA were shifted to the right, in an apparently competitive manner, by well characterized GABA_R-selective antagonists (Fig. 15B). Based on additional experiments, the EC₅₀'s are 1.32 μ M for GABA and 3.31 μ M for baclofen. The results to date are summarized in Table 2. Antagonist affinity estimates (Fig. 15B, Table 2) were similar to values reported in previous electrophysiological studies using brain tissue (Bon, C., et al., 1996; Seabrook, G. R., et al., 1990), as well as to those obtained by measuring displacement of radioligand from cells expressing GABA_R1 alone (Kaupmann, K., et al., 1997a) (Table 2).

Table 2. Agonist and antagonist pharmacology in cells expressing GABA_BR1, GABA_BR2, or both.

<u>Protein</u>		<u>Measurement</u>	<u>Agonist</u>			<u>Antagonist</u>	
		GABA	Baclofen	3-APMPA	Phaclofen	CGP54626	CGP55845
5	GABA _B R1+GABA _B R2	pEC ₅₀ ¹ , pK _B ²	5.88 ±0.01	5.48 ±0.05	7.29 ±0.02	3.80 ± 0.03 ⁴	7.48 ±0.05 8.60 ±0.09
	GABA _B R1	pK _i ³	4.6	4.3	5.2	>3.0	8.95 8.7

¹ n = 6-8 oocytes except for GABA; n = 20 oocytes.

² Measured using GABA as agonist; n = 4-6 oocytes.

³ Displacement of [³H]-CGP54626 from COS-7 cells expressing GABA_BR1; n = 3.4

⁴ IC₅₀ using EC₅₀ concentration of GABA.

Evidence that GABA-induced currents were mediated by GIRK channels included: 1) dependency on elevated external K^+ , 2) strong inward rectification of the current-voltage (I/V) relation, 3) reversal potential (-23.3 mV) close to the predicted equilibrium potential for K^+ (-23 mV), and 4) sensitivity to block by 100 μM Ba^{++} (Figure 8).

Three oocytes were injected with pertussis toxin (2 ng/oocyte) 6 h before voltage clamping. GABA-stimulated currents were abolished in these oocytes (Table 1a and 1b), suggesting that receptor activation of GIRKs was mediated by G-proteins G_i or G_o . Analogous results have been obtained by others expressing D2 dopamine receptors with GIRKs in oocytes (Werner et al. 1996).

GABA responses in co-transfected HEK-293 cells

To verify that both gene products, $GABA_B R1b$ and $GABA_B R2$, are also required for expression of functional $GABA_B$ receptors in mammalian cells, voltage clamp recordings were obtained from HEK-293 cells transiently transfected with various combinations of each gene along with GIRKs. Cells transfected with a combination of $GABA_B R1b$ (B058) and $GABA_B R2$ (B055) plus GIRKs consistently produced large K^+ currents in response to 100 μM GABA (9 of 10 cells tested, Table 1a and 70 of 81 cells tested, Table 1b). Large amplitude currents were also observed when $GABA_B R2$ was paired with the $GABA_B R1a$ splice variant (1046 " 247 pA; n = 9). In contrast, cells transfected with only one of the $GABA_B$ genes plus GIRKs responded either not at all or only very weakly to GABA (Table 1a and 1b). Small agonist-evoked currents (10-50 pA) were observed in 5 of 26 cells expressing $GABA_B R1$; similar weak currents were evoked in 1 of 23 cells expressing $GABA_B R2$.

GABA-elicited currents in doubly transfected cells were completely blocked by 100 μM Ba^{++} or the competitive antagonist CGP55845 at 1 μM (Figure 9). The EC_{50} for GABA stimulation of GIRKs in HEK-293 cells was determined using similar methods as for oocytes. The EC_{50} , 3.42 μM , was comparable to that measured in oocytes (1.76 μM ; further experiments gave 1.32 μM). Thus, whether in *Xenopus* oocytes or HEK-293 cells, the behavior of the GABA_B receptor is the same. Co-expression of both $\text{GABA}_\text{B}\text{R1b}$ and $\text{GABA}_\text{B}\text{R2}$ is required to observe activation of the receptor by GABA.

To determine if co-expressed $\text{GABA}_\text{B}\text{R1/R2}$ could mediate a cellular response in the absence of exogenously supplied GIRKs, we transiently co-transfected CHO cells with $\text{GABA}_\text{B}\text{R1}$ and $\text{GABA}_\text{B}\text{R2}$ and measured agonist-evoked extracellular acidification using a microphysiometer. Baclofen stimulated a 9-fold increase in acidification rate (Fig 16) which was blocked by 100 nM CGP55845 and by pretreatment with PTX (not shown). This response was absent in cells expressing either protein alone. Since GIRK activity is undetectable in wild-type CHO cells (Krapivinsky, G., et al., 1995b) we conclude that GIRK expression is not a prerequisite for signal generation by $\text{GABA}_\text{B}\text{R1/R2}$.

$\text{GABA}_\text{B}\text{R1/GABA}_\text{B}\text{R2}$ signaling through chimeric G-proteins

Chimeric G-proteins have been used to "switch" the coupling pathway of a GPCR from one that normally inhibits adenylyl cyclase to one that activates phospholipase C (Conklin et al., 1993). With the aim of developing an assay based on Ca^{++} or some other signal amenable to high throughput screening, we employed a $\text{Ga}_{q/i3}$ chimera to obtain Ca^{++} -induced Cl^- responses in oocytes. Oocytes were injected with $\text{GABA}_\text{B}\text{R1}$ and $\text{GABA}_\text{B}\text{R2}$ mRNAs as previously described. 2-3 days later oocytes were injected again with 50 pg of

Ga_{q/i3} mRNA and recorded under voltage clamp conditions. In response to GABA (0.1 - 1 mM) 88% of these oocytes produced rapidly desensitizing inward currents (454 ± 92 nA; n = 14) typical of those stimulated by receptors that normally couple to Ga_q. In contrast, oocytes injected with only the GABA_BR1/GABA_BR2 combination (n > 100), or GABA_BR1 plus Ga_{q/i3} (n = 4) failed to produce currents.

GABA_B agonists also resulted in concentration-dependent stimulation of phosphoinositide production in COS-7 cells transfected transiently with GABA_BR1, GABA_BR2, and the chimeric G-protein Ga_{q/z}. The concentration of agonist evoking 50% of its maximum response (EC₅₀) and fold stimulation over basal were: GABA (EC₅₀ = 1.8 μM; 2.4 fold); baclofen (1.7 μM; 1.8 fold); 3-aminopropylmethylphosphinic acid (EC₅₀ = 0.11 μM; 2.2 fold). These results indicate that G-protein chimeras, in particular Ga_{q/z} and Ga_{q/i3}, are useful for directing GABA_B receptor stimulation to a phosphoinositide- or Ca⁺⁺-based assay.

A comparison of the pharmacological properties of GABA_BR1 and GABA_BR2 using radioligand binding revealed that membranes from HEK293 or COS-7 cells expressing GABA_BR1, but not those expressing GABA_BR2, were labeled by the high affinity antagonist [³H]-CGP54626²¹ (Table 2), indicating that the polypeptides are pharmacologically distinct. Neither was labeled by the agonists [³H]-GABA or [³H]-baclofen. Furthermore, with the available ligands (GABA, baclofen, APMPA, phaclofen, CGP54626, CGP-55845 and SCH-50911) the binding profile of membranes from cells co-transfected with GABA_BR1/R2 was not different from those transfected with GABA_BR1 alone. The absence of detectable high affinity agonist binding to GABA_BR1/R2, as well as to GABA_BR1b, constitutes a notable distinction from the GABA_B binding profile in the CNS and may reflect the

absence of an essential, as yet undefined G-protein or accessory protein.

The molecular mechanism by which protein co-expression confers functional activity is unknown. We noted that varying the ratios of GABA_BR1/R2 cDNAs from 1/100 to 100/1 in HEK293 cells resulted in a symmetrical fall off in response amplitude (Fig.14B). This suggests that a 1:1 protein stoichiometry may be critical, and caused us to postulate that the polypeptides are forming a heteromeric association. Biochemical evidence supports the idea that certain GPCRs can exist as homodimers (Hebert, T.E., et al., 1996; Cvejic, S., et al., 1997; Ciruela, F., et al., 1995; Avissar, S., et al., 1983; Romano, C., et al., 1996), but the functional significance of this has been largely unexplored (Hebert, T.E., et al., 1996; Wreggett, K.A., et al., 1995). The possibility of a physical association was investigated using epitope-tagged versions of GABA_BR1 (RGS6xH tag) and GABA_BR2 (HA tag). C-terminal modification did not appear to alter the function of either polypeptide; maximal current amplitudes (Fig. 14B) and EC₅₀ values for GABA (4.97 μ M, n = 5) were unchanged compared to HEK293 cells expressing the wild-type GABA_BR1/R2 receptor combination (3.42 μ M, n = 5). The subcellular distribution of epitope-tagged proteins was examined in transfected cells by fluorescence microscopy. When expressed individually, GABA_BR1^{RGS6xH} and GABA_BR2^{HA} were localized throughout the plasma membrane. Optical sectioning of antibody-labeled cells by confocal microscopy confirmed the membrane localization pattern, with less labeling in the cytoplasm and none in the nucleus. In co-transfected cells there was a striking overlap in the distribution of the two epitope tags (Fig. 17A-17C). Both proteins were prominently expressed on the plasma

membrane. Furthermore, co-localization occurred within the cytoplasm, suggesting that GABA_BR1 and GABA_BR2 assemble in the endoplasmic reticulum. In contrast, the cellular distribution of an unrelated GPCR, NPY Y5, differed considerably from that of GABA_BR2 (Fig. 17D), suggesting specificity in the association of GABA_BR2 with GABA_BR1.

Western blots of whole cell extracts from cells expressing GABA_BR1^{RGS6xH}, GABA_BR2^{HA} or both, exhibited bands close to the predicted molecular weights of the two proteins (92 kD for GABA_BR1, 97 kD for GABA_BR2) and additional bands corresponding to the predicted molecular weights of receptor dimers (Fig. 18A,B). To determine if GABA_BR1 and GABA_BR2 co-associate in a heteromeric complex, we immunoprecipitated solubilized material from cells expressing both polypeptides. GABA_BR2^{HA} was detected in material immunoprecipitated using either anti-His or anti-HA antibodies (Fig. 18). To determine if GABA_BR1b and GABA_BR2 co-associate in a heteromeric complex, we performed immunoprecipitations using membrane fractions enriched in plasma membrane as determined by the presence of Na⁺/K⁺ ATPase (Figure 20A). In co-transfected cells only, GABA_BR2^{HA} was detected in material immunoprecipitated using antibodies specific for the GABA_BR1^{RGS6xH} protein (Figure 20B). This result confirms that both GABA_BR1 and GABA_BR2 are correctly targeted to the plasma membrane of HEK293 cells, and that the two proteins exist in a heteromeric complex, perhaps as heterodimers, on the membrane surface.

Experimental Discussion

A gene has been cloned that shows 38% overall identity at the amino acid level with the recently cloned GABA_BR1 polypeptide. Important predicted features of the new gene product include 7 transmembrane spanning regions, and a large extracellular N-terminal domain. Like the GABA_BR1 gene product, GABA_BR2 by itself does not promote the activation of cellular effectors such as GIRKs.

When co-expressed together, however, the two permit a GABA_B receptor phenotype that is quite similar to that found in the brain. The functional attributes of this reconstituted receptor include: 1) robust stimulation of a physiological effector (GIRKs), 2) EC₅₀s for GABA and baclofen in the same range as for GABA_B receptors previously studied in the CNS, 3) antagonism by the high affinity selective antagonist CGP55845, and 4) inhibition of receptor function by pertussis toxin. These attributes are not observed when either GABA_BR1 or GABA_BR2 is expressed alone.

Our data indicate that GABA_BR1 and GABA_BR2 associate as subunits to produce a single pharmacologically and functionally defined receptor. Consistent with this view, double labeling *in situ* hybridization experiments provided evidence that GABA_BR1 and GABA_BR2 mRNAs are co-expressed in individual neurons and populations of neurons in several regions of the nervous system including hippocampal pyramidal cells (Fig. 21), cerebellar Purkinje cells (Fig. 12A,B) and sensory neurons in mesencephalic trigeminal nucleus (Fig. 21) and dorsal root ganglia. This co-localization pattern of GABA_BR1 and R2 transcripts predicts that GABA_B receptors on these cells are comprised of GABA_BR1/R2 heteromers. Other as yet unidentified GABA_B receptor homologues may associate elsewhere to produce novel

subtypes. For example, the low level of expression of GABA_BR2 mRNA relative to GABA_BR1 in caudate putamen and hypothalamus (Fig. 11A,B) raises the possibility that other GABA_B receptor homologues may associate with GABA_BR1 to produce novel subtypes in these regions. Conclusive evidence that functional GABA_B receptors exist in vivo as multimers will await immunofluorescence studies with specific antibodies.

The recent cloning of a family of accessory proteins that modify the binding and functional properties of a calcitonin-receptor-like receptor (McLarchie, et al., 1998) demonstrates that some 7-TM spanning proteins require additional unrelated proteins to reconstitute native GPCR activity. GABA_BR1 and GABA_BR2 are the first examples of 7-TM proteins for which activity is dependent on an interaction with another member within the same family of proteins. There will be considerable interest in whether other GPCRs are formed by heteromeric complexes of related 7-TM proteins. Many members of the superfamily of GPCRs, such as D₃, 5-HT₅, and olfactory receptors, do not function well in heterologous expression systems and may require related partners to generate native receptor function (Nimischinsky, et al., 1997). The growing list of receptors that have been reported to exist as homodimers (Ciruela, F., et al., 1995; Cvejic, S., et al., 1997; Hebert, T.E., et al., 1996; Romano, C., et al., 1996; Maggio, R., et al., 1996) points to the likelihood that both homomeric and heteromeric assemblies are more widespread among GPCRs than previously thought.

There are several possible explanations for why two genes are required for full function of the GABA_B receptor. One possible explanation is that the two gene products function together as a heterodimer having

high affinity agonist and antagonist binding sites. Currently, there is no precedent for heterodimerization of GPCRs. There is evidence that certain GPCRs, for example the mGluR5 receptor, can form homodimers via cystine disulfide bridges in the N-terminal domain (Romano et al., 1996). Significantly, synthetic peptides that inhibit homodimerization of beta2-adrenergic receptors also reduce agonist stimulation of adenylyl cyclase activity (Hebert et al., 1996). Useful parallels may be drawn from other classes of receptors where heterodimeric structures are well-known. For example, the NMDA (glutamate) receptor is comprised of two principal subunits, neither of which alone permits all of the native features of the receptor (see Wisden and Seeburg, 1993). GABA_B receptors may be comprised similarly of two (or more) peptide subunits, such as GABA_BR1 and GABA_BR2, that form a quaternary structure having appropriate binding sites for agonist and G-protein.

A role for GABA_BR2 in modulating sensory information is suggested by *in situ* hybridization histochemistry which revealed the expression of GABA_BR2 mRNA in relay nuclei of several sensory pathways. In the olfactory and visual pathways GABA_BR2 appears to be in a position to modulate excitatory glutamatergic projections from the olfactory bulb and retina. GABA_BR2 mRNA was observed in the target regions of projection fibers from the main olfactory bulb, including the olfactory tubercle, piriform and entorhinal cortices and from the retina, for instance the superior colliculus (Figures 19A,B; Table 3).

The ability to modulate nociceptive information might be indicated not only by the presence of GABA_BR2 transcripts in somatic sensory neurons of the

trigeminal and dorsal root ganglia (Figures 19H-I) but also by being present in the target regions of nociceptive primary afferent fibers, including the superficial layers of the spinal trigeminal nucleus and dorsal horn of the spinal cord (Figures 19F-G). Again, in each of these loci GABA_BR2 has been shown to be in a position to potentially modulate the influence of excitatory glutamatergic nociceptive primary afferents. In both ganglia, microscopic examination indicated that the hybridization signal did not appear to be restricted to any one size cell and was distributed evenly over small, medium and large ganglion cells. Thus, GABA_BR2 may be able to influence various sensory modalities. Expression levels appeared to be higher in the ganglion cells of the dorsal root with light to moderate expression in the trigeminal ganglia.

GABA_BR2 mRNA was likewise observed to be expressed in the vestibular nuclei which are target regions of inhibitory GABAergic Purkinje cells and also in the Purkinje cells themselves, suggesting that GABA_BR2 may be important in the mediation of planned movements (Figure 19F).

Moderate expression of GABA_BR2 transcripts throughout the telencephalon indicate a potential modulatory role in the processing of somatosensory and limbic system (entorhinal cortex) information, in addition to modulating visual (parietal cortex) and auditory stimuli (temporal cortex) as well as cognition. Furthermore, modulation of patterns of integrated behaviors, such as defense, ingestion, aggression, reproduction and learning could also be attributed to this receptor owing to its expression in the amygdala (Table 3). The high levels of expression in the thalamus suggest a possible regulatory role in the

Table 3. Distribution of rGABA_BR2, rGABA_BR1a, and GABA_B1b mRNA in the rat CNS. The strength of the hybridization signal for each of the respective mRNAs obtained in various regions of the rat brain was graded as weak (+), moderate (++) ,heavy (+++) or intense (++++)and is relative to the individual polypeptides.

Region	GABA _B R2	GABA _B R1a*	GABA _B R1b*	Potential Application
Olfactory bulb				Modulation of olfactory sensation
internal granule layer	+	++	++	
glomerular layer	+	++	++	
external plexiform layer	-	-	-	
mitral cell layer	-	+	++	
anterior olfactory n	++	++	++	
olfactory tubercle	+	++	+++	
Islands of Calleja	-	++	+++	
Telencepha- lon				Sensory integration
taenia tecta	++	++	++	
frontal cortex	++	++	++	
orbital cortex	++	++	++	
agranular insular cortex	+++	++	++	

	Region	GABA _B R2	GABA _B R1a*	GABA _B R1b*	Potential Application
5	cingulate cortex	++	++	+	
	retrosplenial cortex	++	++	+	
	parietal cortex	++	++	++	Processing of visual stimuli
10	occipital cortex	++	++	++	
	temporal cortex	++	++	++	Processing of auditory stimuli
	perirhinal cortex	++	++		
15	entorhinal cortex	++	++	++	Processing of visceral information
	dorsal endopiriform	++	++	++	
20	piriform cortex	+++	+++	+++	Integration/transmission of incoming olfactory information
	B a s a l Ganglia				
25	accumbens n	+	++	++	Modulation of dopaminergic function
	caudate-putamen	+	+	++	Sensory/motor integration
	globus pallidus	+	-	+	

Septum				
Region	GABA _B R2	GABA _B R1a*	GABA _B R1b*	Potential Application
medial septum	++	++	+	Cognitive enhancement via cholinergic system
lateral septum	++	+	++	Modulation of integration of stimuli associated with adaptation
septohippocampal n	+	+	+++	
diagonal band n	++	++	++	
ventral pallidum	++	+	+	
Amygdala				Anxiolytic (activation - reduction in panic attacks) appetite, depression
basolateral n	++	+	+	
medial amygdaloid n	+	+	+	Olfactory amygdala
basomedial n	+	+		
central n	+++	-	+	
anterior cortical n	+	+	+	
postero-medial cortical n	++	+	+	
bed n stria terminalis	++	+	++	

Region	GABA _B R2	GABA _B R1a*	GABA _B R1b*	Potential Application
zona incerta	+	+	+	
Hippocampus				Memory consolidation and retention
CA1, Ammon's horn	++	+++	+++	
CA2, Ammon's horn	++++	+++	+++	
CA3, Ammon's horn	++++	+++	+++	Facilitation of LTP
subiculum	+	+++	+++	
parasub-iculum	++	++	++	
presubiculum	++	++	++	
dentate gyrus	++++	+++	++	
polymorph dentate gyrus	+++	+++	++	
Hypothalamus				
suprachiasmatic n	+	++	ND	
median preoptic area	+	+	++	Regulation of gonadotropin secretion and reproductive behaviors
paraventricular n	+	++	++	Appetite/obesity
arcuate n	++	++	++	
anterior hypoth, post	+	+		
lateral hypoth	+	+	++	
ventromedial n	+	++	+++	
periventricular n	+	+	+	
supraoptic n	+	++	+	Synthesis of OXY and AVP

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	Region	GABA _B R2	GABA _B R1a*	GABA _B R1b*	Potential Application
5	supramam-millary n	++	++	++	Modulation of hypothalamic projections to cortex
	premam-millary n	+	+	+	
	medial mammillary n	+	++	+	
10	Thalamus				Analgesia/Modulation of sensory information
	paraventricular n	++	+	++	Modulation of motor and behavioral responses to pain
	centromedial n	++	+	++	Modulation of motor and behavioral responses to pain
15	paracentral n.	++	+	++	
	parafascicular n	++	+	++	Modulation of motor and behavioral responses to pain
20	anterodorsal n	+++	+	++	Modulation of eye movement
	laterodorsal n	+++	+	++	
	lateral posterior n	++	+	++	

Region	GABA _B R2	GABA _B R1a*	GABA _B R1b*	Potential Application
reuniens n	+++	+	++	Modulation of thalamic input to ventral hippocampus and entorhinal ctx
rhomboid n	+++	+	++	
m e d i a l habenula	++++	+	++++	Anxiety/sleep disorders/ analgesia in chronic pain
l a t e r a l habenula	+	+	+++	
ventrolateral n	+++	+	++	
ventromedial n	+++	++	++	
ventral posterolateral n	+++	+	++	
reticular n	++	+	+	Alertness /sedation
l a t e r a l geniculate n	++	+	++	Modulation of visual perception
m e d i a l geniculate	++	+	++	Modulation of auditory system
subthalamic n	++	++	++	
Mesencephalon				
superior colliculus	+	+	+	Modulation of vision
inferior colliculus	+	+	+	
central gray	+	+	+	Analgesia
dorsal raphe	+	++	+	

	Region	GABA _B R2	GABA _B R1a*	GABA _B R1b*	Potential Application
5	d e e p mesence - phalic n	+	+	+	
	oculomotor n	+			
	pontine n	+++		++	
10	retrobulbar field	+			
	ventral tegmental area	+	++	++	Modulation of the integration of motor behavior and adaptive responses
15	substantia nigra , reticular	+	+	+	Motor control
	substantia nigra , compact	++	++	++	
20	interpedunc ular n	++	ND	ND	Analgesia
	Myelence - phalon				Analgesia
25	raphe magnus	++		++	
	raphe pallidus	+	++	ND	
	principal trigeminal	+	+		
30	spinal trigeminal n	+	+	+	
	pontine reticular n	++	+	++	
35	parvocell- ular reticular n	+	++	++	

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	Region	GABA _B R2	GABA _B R1a*	GABA _B R1b*	Potential Application
5	l o c u s coeruleus	++	++	++	Modulation of NA transmission
	parabrachial n	+	++	+	Modulation of visceral sensory information
	vestibular n	+	++	+	Maintenance of balance and equilibrium
10	gigantocell- u l a r reticular n	+	++	++	Inhibition and disinhibition of brainstem
15	prepositus hypoglossal n	+	+++	++	Position and movement of the eyes/ Modulation of arterial pressure and heart rate
	v e n t r a l cochlear n	++	+	ND	
	n s o l t a r y tract	++			Hypertension
20	A5 Nor- adrenaline cells	+	ND	ND	
25	facial n(7)	+	++	+	
	Cerebellum				Motor coordina- tion, Autism
	granule cell layer	+	+	+	
	P u r k i n j e cells	++	-	++	

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Region	GABA _B R2	GABA _B R1a*	GABA _B R1b*	Potential Application
Spinal cord				Analgesia
dorsal horn	+	++	+	
ventral horn	+	++	+	
trigeminal ganglion	++	+++	+	Nociception
dorsal root ganglion	++++	+++	ND	Nociception

ND = not determined

*Bischoff S et al.

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List of Abbreviations

7	facial n
ac	anterior commissure
Acb	accumbens n
ACo	anterior cortical amygdaloid n
AI	agranular insular cortex
AON	anterior olfactory n
APir	amygdalopiriform transition area
APT	anterior pretectal n
Arc	arcuate hypothalamic n
BLA	basolateral amygdaloid n
CA1-3	Fields of Ammon's horn
cc	corpus callosum
Cg	cingulate cortex
CeA	central amygdaloid n
CPu	caudate-putamen
DG	dentate gyrus
DLG	dorsal lateral geniculate n
DpMe	deep mesencephalic n
Ent	entorhinal cortex
Gi	gigantocellular reticular n
Gr	granule cell layer, cerebellum
GrO	granule layer olf. bulb
FrA	frontal association cortex
GP	globus pallidus
HDB	horizontal diagonal band
LA	lateral amygdaloid n
LH	lateral hypothalamus
LO	lateral orbital cortex
LV	lateral ventricle
M1	primary motor cortex
MeAD	medial amygdaloid n, anterodorsal
MG	medial geniculate

MHb	medial habenular n
MPO	medial preoptic n
PC	Purkinje cell layer of the cerebellum
PF	parafascicular n
Pir	piriform cortex
PMCo	posteromedial cortical amygdaloid n
Pr	prepositus n
PVA	paraventricular thalamic n
RS	retrosplenial cortex
S	subiculum
SFi	septofofimbrial n
SI	substantia innominata
SNC	substantia nigra,compact
STh	subthalamic n
Sp5	spinal trigeminal n
TT	tenia tecta
Ve	vestibular n
VTA	ventral tegmental area

Potential therapeutic application for GABA_B agonists and antagonists

Agonists

Antinociception

A potential GABA_B agonist application may in antinociception. The inhibitory effects of GABA and GABA_B agonists are thought to be predominantly a presynaptic mechanism on excitation-induced impulses in high threshold Ad and C fibers on primary afferents. This effect can be blocked by GABA_B antagonists (Hao, J-H., et al., 1994). Baclofen's spinal cord analgesic effects have been well documented in the rat, though it has not been as effective in human. However, baclofen

has been successful in the treatment of trigeminal neuralgia in human.

The localization of the GABA_BR2 mRNA in the superficial layers of the spinal cord dorsal horn, the termination site for primary afferents, as well as their cells of origin in the dorsal root and trigeminal ganglia position the GABA_BR1/R2 receptor appropriately for mediating the agonist effects.

Drug Addiction

It has been suggested that GABA agonists may have some potential in the treatment of cocaine addiction. A role for the action of psychostimulants in the mesoaccumbens dopamine system is well established. The ventral pallidum receives a GABAergic projection from the nucleus accumbens and both regions contain GABA_BR2 transcripts. GABA receptors were shown to have an inhibitory effect on dopamine release in the ventral pallidum. Phaclofen acting at these receptors resulted in increased dopamine release and baclofen was shown to attenuate the reinforcing effects of cocaine. (Roberts, D. C. S., et al., 1996; Morgan, A.E. et al.)

Micturition

There is a potential application for GABA_B agonists in the treatment of bladder dysfunction. Baclofen has been used in the treatment of detrusor hyperreflexia through inhibition of contractile responses. In addition to a peripheral site of action for GABA_B agonists, there is also the possibility for a central site. The pontine micturition center in the brainstem is involved in mediating the spinal reflex pathway, via Onuf's nucleus in the sacral spinal cord. Support for

possible application of GABA_B agonists in the treatment of bladder dysfunction may be augmented by presence of GABA_BR2 mRNA in the various nuclei involved in the control of the lower urinary tract function.

Antagonists

Memory Enhancement - Alzheimer's Disease

GABA_B antagonists may have a potential application in the treatment of Alzheimer's Disease. The blockade of GABA_B receptors might lead to signal amplification and improvement in cognitive functions resulting from an increased excitability of cortical neurons via amplification of the acetylcholine signal. Additionally, memory may be enhanced by GABA_B antagonists which have been shown to suppress late IPSPs, thus facilitating long-term potentiation in the hippocampus (see Table 3).

To support this idea, CGP36742, a GABA_B antagonist, has been shown to improve learning performance in aged rats as well as the performance of rhesus monkeys in conditioned spatial color task. (Mondadori, C. et al., 1993). The significance of the GABA_BR1/R2 receptor in cognitive functioning might be indicated by the presence of GABA_BR2 mRNA in the cerebral cortex and its codistribution in the ventral forebrain with cortically projecting cholinergic neurons as well as its localization in the pyramidal cells in all regions of Ammon's horn and dentate gyrus in the hippocampus.

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[Published erratum appears in Neuron 1997 Oct. 19(4):
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SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Jones, Kenneth A.
Laz, Thomas M.
Borowsky, Beth
- (ii) TITLE OF INVENTION: DNA Encoding a GABABR2 Polypeptide And
Uses Thereof
- (iii) NUMBER OF SEQUENCES: 47
- (iv) CORRESPONDENCE ADDRESS:
 - (A) ADDRESSEE: Cooper & Dunham LLP
 - (B) STREET: 1185 Avenue of the Americas
 - (C) CITY: New York
 - (D) STATE: New York
 - (E) COUNTRY: U.S.A.
 - (F) ZIP: 10036
- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version #1.30
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER: Not Yet Known
 - (B) FILING DATE: Herewith
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: White Esq., John P.
 - (B) REGISTRATION NUMBER: 28,678
 - (C) REFERENCE/DOCKET NUMBER: 54002-D
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: 212-278-0400
 - (B) TELEFAX: 212-391-0525

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 3244 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

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(2) INFORMATION FOR SEQ ID NO:2:

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- (A) LENGTH: 898 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:
- | | | | | | | | | | | | | | | | |
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(2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 2823 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
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- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

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GGGGGCATGC TGCTCATCGA CCTGTGCATC CTGATCTGTT GGCAGGCTGT GGACCCCTG	1860
CGGAGGACAG TAGAGAGGTA CAGCATGGAG CCGGACCCAG CAGGCCGGGA CATCTCCATC	1920
CGCCCATTCG TGGAACACTG CGAAAACACC CACATGACCA TCTGGCTTGG CATTGTCTAC	1980
GCCTACAAGG GGCTCCTCAT GCTATTCGGT TGTTTCTTGG CATGGGAAAC CCGCAATGTG	2040
AGCATCCCTG CCCTCAACGA CAGCAAGTAC ATCGGCATGA GTGTGTACAA TGTGGGGATC	2100

ATGTGCATCA TCGGGGCTGC TGTCTCCTTC CTGACGCGTG ACCAGCCCAA CGTGCAGTTC 2160
 TGCATCGTGG CCCTGGTCAT CATCTTCTGC AGCACCATCA CTCTCTGCCT GGTGTTTGTG 2220
 CCAAAGCTCA TTA CTCTGAG GACAAACCCT GACGCAGCCA CTCAGAACAG GCGGTTCCAG 2280
 TTCACACAGA ACCAGAAGAA AGAAGATTCG AAGACCTCCA CTTCAGTCAC CAGCGTGAAC 2340
 CAGGCGAGCA CGTCACGCCT GGAGGGACTG CAGTCAGAAA ACCACCGCCT TCGAATGAAG 2400
 ATCACAGAGC TGGACAAAGA CTTGGAAGAA GTCACCATGC AGCTACAAGA CACACCAGAG 2460
 AAGACCACAT ACATCAAACA GAATCACTAC CAAGAGCTCA ACGACATCCT CAGCTTGGGC 2520
 AACTTCACAG AGAGCACAGA TGGAGGAAAG GCCATTCTAA AAAATCACCT CGATCAAAAC 2580
 CCCCAGCTCC AGTGGAACAC GACAGAGCCC TCAAGAACAT GCAAAGACCC CATAGAAGAC 2640
 ATCAACTCCC CGGAGCACAT CCAGCGCCGG CTGTCGCTCC AGCTCCCCAT CCTTCACCAC 2700
 GCCTACCTCC CATCCATCGG AGGCGTGGAT GCCAGCTGCG TCAGCCCCTG TGTCAGCCCT 2760
 ACCGCCAGCC CTCGCCACAG ACACGTACCA CCCTCCTTCC GAGTCATGGT CTCGGGCCTG 2820
 TAG 2823

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 940 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met Ala Ser Pro Pro Ser Ser Gly Gln Pro Arg Pro Pro Pro Pro Pro
 1 5 10 15
 Pro Pro Pro Ala Arg Leu Leu Leu Pro Leu Leu Leu Ser Leu Leu Leu
 20 25 30
 Trp Leu Ala Pro Gly Ala Trp Gly Trp Thr Arg Gly Ala Pro Arg Pro
 35 40 45
 Pro Pro Ser Ser Pro Pro Leu Ser Ile Met Gly Leu Met Pro Leu Thr
 50 55 60
 Lys Glu Val Ala Lys Gly Ser Ile Gly Arg Gly Val Leu Pro Ala Val
 65 70 75 80
 Glu Leu Ala Ile Glu Gln Ile Arg Asn Glu Ser Leu Leu Arg Pro Tyr
 85 90 95
 Phe Leu Asp Leu Arg Leu Tyr Asp Thr Glu Cys Asp Asn Ala Lys Gly

145

100										105					110				
Leu	Lys	Ala	Phe	Tyr	Asp	Ala	Ile	Lys	Tyr	Gly	Pro	Asn	His	Leu	Met				
		115					120					125							
Val	Phe	Gly	Gly	Val	Cys	Pro	Ser	Val	Thr	Ser	Ile	Ile	Ala	Glu	Ser				
	130					135					140								
Leu	Gln	Gly	Trp	Asn	Leu	Val	Gln	Leu	Ser	Phe	Ala	Ala	Thr	Thr	Pro				
145					150					155					160				
Val	Leu	Ala	Asp	Lys	Lys	Lys	Tyr	Pro	Tyr	Phe	Phe	Arg	Thr	Val	Pro				
				165					170					175					
Ser	Asp	Asn	Ala	Val	Asn	Pro	Ala	Ile	Leu	Lys	Leu	Leu	Lys	His	Phe				
			180					185					190						
Arg	Trp	Arg	Arg	Val	Gly	Thr	Leu	Thr	Gln	Asp	Val	Gln	Arg	Phe	Ser				
		195					200					205							
Glu	Val	Arg	Asn	Asp	Leu	Thr	Gly	Val	Leu	Tyr	Gly	Glu	Asp	Ile	Glu				
	210					215					220								
Ile	Ser	Asp	Thr	Glu	Ser	Phe	Ser	Asn	Asp	Pro	Cys	Thr	Ser	Val	Lys				
225					230					235					240				
Lys	Leu	Lys	Gly	Asn	Asp	Val	Arg	Ile	Ile	Leu	Gly	Gln	Phe	Asp	Gln				
				245					250					255					
Asn	Met	Ala	Ala	Lys	Val	Phe	Cys	Cys	Ala	Phe	Glu	Glu	Ser	Met	Phe				
			260					265					270						
Gly	Ser	Lys	Tyr	Gln	Trp	Ile	Ile	Pro	Gly	Trp	Tyr	Glu	Pro	Ala	Trp				
		275				280						285							
Trp	Glu	Gln	Val	His	Val	Glu	Ala	Asn	Ser	Ser	Arg	Cys	Leu	Arg	Arg				
	290					295					300								
Ser	Leu	Leu	Ala	Ala	Met	Glu	Gly	Tyr	Ile	Gly	Val	Asp	Phe	Glu	Pro				
305					310					315					320				
Leu	Ser	Ser	Lys	Gln	Ile	Lys	Thr	Ile	Ser	Gly	Lys	Thr	Pro	Gln	Gln				
				325					330					335					
Tyr	Glu	Arg	Glu	Tyr	Asn	Ser	Lys	Arg	Ser	Gly	Val	Gly	Pro	Ser	Lys				
			340					345					350						
Phe	His	Gly	Tyr	Ala	Tyr	Asp	Gly	Ile	Trp	Val	Ile	Ala	Lys	Thr	Leu				
		355					360					365							
Gln	Arg	Ala	Met	Glu	Thr	Leu	His	Ala	Ser	Ser	Arg	His	Gln	Arg	Ile				
	370					375					380								
Gln	Asp	Phe	Asn	Tyr	Thr	Asp	His	Thr	Leu	Gly	Lys	Ile	Ile	Leu	Asn				
385					390					395					400				
Ala	Met	Asn	Glu	Thr	Asn	Phe	Phe	Gly	Val	Thr	Gly	Gln	Val	Val	Phe				
				405					410					415					
Arg	Asn	Gly	Glu	Arg	Met	Gly	Thr	Ile	Lys	Phe	Thr	Gln	Phe	Gln	Asp				
			420					425					430						
Ser	Arg	Glu	Val	Lys	Val	Gly	Glu	Tyr	Asn	Ala	Val	Ala	Asp	Thr	Leu				

00445460

435					440					445					
Glu	Ile	Ile	Asn	Asp	Thr	Ile	Arg	Phe	Gln	Gly	Ser	Glu	Pro	Pro	Lys
450						455					460				
Asp	Lys	Thr	Ile	Ile	Leu	Glu	Gln	Leu	Arg	Lys	Ile	Ser	Leu	Pro	Leu
465					470					475					480
Tyr	Ser	Ile	Leu	Ser	Ala	Leu	Thr	Ile	Leu	Gly	Met	Ile	Met	Ala	Ser
				485					490					495	
Ala	Phe	Leu	Phe	Phe	Asn	Ile	Lys	Asn	Arg	Asn	Gln	Lys	Leu	Ile	Lys
			500					505					510		
Met	Ser	Ser	Pro	Tyr	Met	Asn	Asn	Leu	Ile	Ile	Leu	Gly	Gly	Met	Leu
		515					520					525			
Ser	Tyr	Ala	Ser	Ile	Phe	Leu	Phe	Gly	Leu	Asp	Gly	Ser	Phe	Val	Ser
	530					535					540				
Glu	Lys	Thr	Phe	Glu	Thr	Leu	Cys	Thr	Val	Arg	Thr	Trp	Ile	Leu	Thr
545					550					555					560
Val	Gly	Tyr	Thr	Thr	Ala	Phe	Gly	Ala	Met	Phe	Ala	Lys	Thr	Trp	Arg
				565					570					575	
Val	His	Ala	Ile	Phe	Lys	Asn	Val	Lys	Met	Lys	Lys	Lys	Ile	Ile	Lys
			580					585					590		
Asp	Gln	Lys	Leu	Leu	Val	Ile	Val	Gly	Gly	Met	Leu	Leu	Ile	Asp	Leu
		595					600					605			
Cys	Ile	Leu	Ile	Cys	Trp	Gln	Ala	Val	Asp	Pro	Leu	Arg	Arg	Thr	Val
	610					615					620				
Glu	Arg	Tyr	Ser	Met	Glu	Pro	Asp	Pro	Ala	Gly	Arg	Asp	Ile	Ser	Ile
625					630					635					640
Arg	Pro	Leu	Leu	Glu	His	Cys	Glu	Asn	Thr	His	Met	Thr	Ile	Trp	Leu
				645					650					655	
Gly	Ile	Val	Tyr	Ala	Tyr	Lys	Gly	Leu	Leu	Met	Leu	Phe	Gly	Cys	Phe
			660					665					670		
Leu	Ala	Trp	Glu	Thr	Arg	Asn	Val	Ser	Ile	Pro	Ala	Leu	Asn	Asp	Ser
		675					680					685			
Lys	Tyr	Ile	Gly	Met	Ser	Val	Tyr	Asn	Val	Gly	Ile	Met	Cys	Ile	Ile
	690					695					700				
Gly	Ala	Ala	Val	Ser	Phe	Leu	Thr	Arg	Asp	Gln	Pro	Asn	Val	Gln	Phe
705					710					715					720
Cys	Ile	Val	Ala	Leu	Val	Ile	Ile	Phe	Cys	Ser	Thr	Ile	Thr	Leu	Cys
				725					730					735	
Leu	Val	Phe	Val	Pro	Lys	Leu	Ile	Thr	Leu	Arg	Thr	Asn	Pro	Asp	Ala
			740					745					750		
Ala	Thr	Gln	Asn	Arg	Arg	Phe	Gln	Phe	Thr	Gln	Asn	Gln	Lys	Lys	Glu
		755					760					765			
Asp	Ser	Lys	Thr	Ser	Thr	Ser	Val	Thr	Ser	Val	Asn	Gln	Ala	Ser	Thr

770	775	780
Ser Arg Leu Glu Gly Leu Gln Ser Glu Asn His Arg Leu Arg Met Lys 785 790 795 800		
Ile Thr Glu Leu Asp Lys Asp Leu Glu Glu Val Thr Met Gln Leu Gln 805 810 815		
Asp Thr Pro Glu Lys Thr Thr Tyr Ile Lys Gln Asn His Tyr Gln Glu 820 825 830		
Leu Asn Asp Ile Leu Ser Leu Gly Asn Phe Thr Glu Ser Thr Asp Gly 835 840 845		
Gly Lys Ala Ile Leu Lys Asn His Leu Asp Gln Asn Pro Gln Leu Gln 850 855 860		
Trp Asn Thr Thr Glu Pro Ser Arg Thr Cys Lys Asp Pro Ile Glu Asp 865 870 875 880		
Ile Asn Ser Pro Glu His Ile Gln Arg Arg Leu Ser Leu Gln Leu Pro 885 890 895		
Ile Leu His His Ala Tyr Leu Pro Ser Ile Gly Gly Val Asp Ala Ser 900 905 910		
Cys Val Ser Pro Cys Val Ser Pro Thr Ala Ser Pro Arg His Arg His 915 920 925		
Val Pro Pro Ser Phe Arg Val Met Val Ser Gly Leu 930 935 940		

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 45 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: DNA (genomic)
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

AGGGATGCTT TCCTATGCTT CCATATTTCT CTTGGCCTT GATGG

45

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 45 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CAATGTGCAG TTCTGCATCG TGGCTCTGGT CATCATCTTC TGCAG

45

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CTTCTAGGCC TGTACGGAAG TGTT

24

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GTTGTGGTTT GTCCAAACTC ATCAAT

26

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

24

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(i) SEQUENCE CHARACTERISTICS:
    (A) LENGTH: 26 base pairs
    (B) TYPE: nucleic acid
    (C) STRANDEDNESS: single
    (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

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26

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(i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 26 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:
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26

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(i) SEQUENCE CHARACTERISTICS:
      (A) LENGTH: 26 base pairs
      (B) TYPE: nucleic acid
      (C) STRANDEDNESS: single
      (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:
```

26

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 26 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

TGACGCAAGA CGTTCAGAGG TTCTCT

26

(2) INFORMATION FOR SEQ ID NO:14:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

TGTAGCCTTC CATGGCAGCA AGCAGA

26

(2) INFORMATION FOR SEQ ID NO:15:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

AGAGAACCTC TGAACGTCTT GCGTCA

26

(2) INFORMATION FOR SEQ ID NO:16:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iv) ANTI-SENSE: NO

GGCTCTGTTG TGTTCCACTG TAGCTG

26

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 26 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEO ID NO:17:

TCATGCCGCT CACCAAGGAG GTGGCC

26

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 26 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GGCCACCTCC TTGGTGAGCG GCATGA

26

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TGAGTGAGCA GAGTCCAGAG CCGT

24

(2) INFORMATION FOR SEQ ID NO:20:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

ATGGATGGGA GGTAGGCGTG GTGGAG

26

(2) INFORMATION FOR SEQ ID NO:21:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

CTCTCTGCCC TCACCATCCT CGGGAT

26

(2) INFORMATION FOR SEQ ID NO:22:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GACTCCGGCT CGAATACCAG GCAGAG

26

(2) INFORMATION FOR SEQ ID NO:23:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

CCATGTTTGC AAAGACCTGG AGGGTCC

27

(2) INFORMATION FOR SEQ ID NO:24:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GGTCACGCGT CAGGAAAGAG ACAGCAG

27

(2) INFORMATION FOR SEQ ID NO:25:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

AAGCTTCTAG AGATCCCTCG ACCTC

25

(2) INFORMATION FOR SEQ ID NO:26:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

AGGCGCAGAA CTGGTAGGTA TGGAA

25

(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

CTTCTAGGCC TGTACGGAAG TGTTA

25

(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GTTGTGGTTT GTCCAAACTC ATCAATG

27

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 27 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

CTGCTGTCTC TTCCTGACG CGTGACC

27

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 59 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

CCAAGCTTCT AATACGACTC ACTATAGGGG AGACCATGGG CCCGGGGGGA CCCTGTACC

59

(2) INFORMATION FOR SEQ ID NO:31:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

CACTTGTAAG GCAAATGTAC TCGACTCC

28

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 37 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

CGCGGATCCA TTATGTCTGC ACTCCGAAGG AAATTTG

37

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 38 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

CGCGAATTCT TATGTGAAGC GATCAGAGTT CATTTTTC

38

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 34 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GCGGGATCCG CTATGGCTGG TGATTCTAGG AATG

34

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 29 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: other nucleic acid
- (iii) HYPOTHETICAL: NO
- (iv) ANTI-SENSE: NO
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CCGGAATTCC CCTCACACCG AGCCCCTGG

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:

CGCGGATCCA TTATGTCTGC ACTCCGAAGG AAATTTG

(2) INFORMATION FOR SEQ ID NO:39:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 40 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

CTGAGATGGT CTTGATTTGT TTGGAGCTCA GGGGCTCAAA

40

(2) INFORMATION FOR SEQ ID NO:40:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 43 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

AAGGCCATCA ACTTCCTGCC TGTGGACTAT GAGATCGAAT ATG

43

(2) INFORMATION FOR SEQ ID NO:41:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 43 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

CATATTCGAT CTCATAGTCC ACAGGCAGGA AGTTGATGGC CTT

43

CTGAGATGGT CTTGATTTGT TTGGAGCTCA GGGGCTCAAA

TGGCCGCTGC CTCTTCTGCT GGTGATGGCG GCTGGGGT

38

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

TGGCCGCTGC CTCTTCTGCT GGTGATGGCG GCTGGGGT

ACCCAGCCG CCATCACCAG CAGAAGAGGC AGCGGCCA

38

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

ACCCAGCCG CCATCACCAG CAGAAGAGGC AGCGGCCA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

CCTTGGCTTT GGCCTTGAAC AAGACGTCTG GAGGAGGTGG TCGTT

45

(2) INFORMATION FOR SEQ ID NO:45:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 45 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: YES

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

AACGACCACC TCCTCCAGAC GTCTTGTTCA AGGCCAAAGC CAAGG

45

(2) INFORMATION FOR SEQ ID NO:46:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2826 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

ATGGCTTCCC CGCGGAGCTC CGGGCAGCCC GGGCCGCCGC CGCCGCCGCC ACCGCCGCC	60
GCGCGCCTGC TACTGCTACT GCTGCTGCCG CTGCTGCTGC CTCTGGCGCC CGGGGCCTGG	120
GGCTGGGCGC GGGGCGCCCC CCGGCCGCCG CCCAGCAGCC CGCCGCTCTC CATCATGGGC	180
CTCATGCCGC TCACCAAGGA GGTGGCCAAG GGCAGCATCG GGCGCGGTGT GCTCCCCGCC	240
GTGGAAGTGG CCATCGAGCA GATCCGCAAC GAGTCACTCC TGCGCCCTA CTTCTCTGAC	300
CTGCGGCTCT ATGACACGGA GTGCGACAAC GCAAAGGGT TGAAAGCCTT CTACGATGCG	360
ATAAAATACG GGCCGAACCA CTTGATGGTG TTTGGAGGCG TCTGTCCATC CGTCACATCC	420
ATCATTCAG AGTCCCTCCA AGGCTGGAAT CTGGTGCAGC TTTCTTTTGC TGCAACCACG	480
CCTGTTCTAG CCGATAAGAA AAAATACCCT TATTTCTTTC GGACCGTCCC ATCAGACAAT	540
GCGGTGAATC CAGCCATTCT GAAGTTGCTC AAGCACTACC AGTGAAGCG CGTGGGCACG	600

[illegible]

GGAAACTTCA CTGAGAGCAC AGATGGAGGA AAGGCCATTT TAAAAATCA CCTCGATCAA 2580
 AATCCCCAGC TACAGTGGAA CACAACAGAG CCCTCTCGAA CATGCAAAGA TCCTATAGAA 2640
 GATATAAACT CTCCAGAACA CATCCAGCGT CGGCTGTCCC TCCAGCTCCC CATCCTCCAC 2700
 CACGCCTACC TCCCATCCAT CGGAGGCGTG GACGCCAGCT GTGTCAGCCC CTGCGTCAGC 2760
 CCCACCGCCA GCCCCGCCA CAGACATGTG CCACCCTCCT TCCGAGTCAT GGTCTCGGGC 2820
 CTGTAA 2826

(2) INFORMATION FOR SEQ ID NO:47:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 941 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: not relevant

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

Met	Ala	Ser	Pro	Arg	Ser	Ser	Gly	Gln	Pro	Gly	Pro	Pro	Pro	Pro	Pro	1	5	10	15
Pro	Pro	Pro	Pro	Ala	Arg	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Pro	Leu	Leu	20	25	30	
Leu	Pro	Leu	Ala	Pro	Gly	Ala	Trp	Gly	Trp	Ala	Arg	Gly	Ala	Pro	Arg	35	40	45	
Pro	Pro	Pro	Ser	Ser	Pro	Pro	Leu	Ser	Ile	Met	Gly	Leu	Met	Pro	Leu	50	55	60	
Thr	Lys	Glu	Val	Ala	Lys	Gly	Ser	Ile	Gly	Arg	Gly	Val	Leu	Pro	Ala	65	70	75	80
Val	Glu	Leu	Ala	Ile	Glu	Gln	Ile	Arg	Asn	Glu	Ser	Leu	Leu	Arg	Pro	85	90	95	
Tyr	Phe	Leu	Asp	Leu	Arg	Leu	Tyr	Asp	Thr	Glu	Cys	Asp	Asn	Ala	Lys	100	105	110	
Gly	Leu	Lys	Ala	Phe	Tyr	Asp	Ala	Ile	Lys	Tyr	Gly	Pro	Asn	His	Leu	115	120	125	
Met	Val	Phe	Gly	Gly	Val	Cys	Pro	Ser	Val	Thr	Ser	Ile	Ile	Ala	Glu	130	135	140	
Ser	Leu	Gln	Gly	Trp	Asn	Leu	Val	Gln	Leu	Ser	Phe	Ala	Ala	Thr	Thr	145	150	155	160
Pro	Val	Leu	Ala	Asp	Lys	Lys	Lys	Tyr	Pro	Tyr	Phe	Phe	Arg	Thr	Val	165	170	175	
Pro	Ser	Asp	Asn	Ala	Val	Asn	Pro	Ala	Ile	Leu	Lys	Leu	Leu	Lys	His				

515					520					525					
Leu	Ser	Tyr	Ala	Ser	Ile	Phe	Leu	Phe	Gly	Leu	Asp	Gly	Ser	Phe	Val
530						535					540				
Ser	Glu	Lys	Thr	Phe	Glu	Thr	Leu	Cys	Thr	Val	Arg	Thr	Trp	Ile	Leu
545					550					555					560
Thr	Val	Gly	Tyr	Thr	Thr	Ala	Phe	Gly	Ala	Met	Phe	Ala	Lys	Thr	Trp
				565					570					575	
Arg	Val	His	Ala	Ile	Phe	Lys	Asn	Val	Lys	Met	Lys	Lys	Lys	Ile	Ile
			580					585						590	
Lys	Asp	Gln	Lys	Leu	Leu	Val	Ile	Val	Gly	Gly	Met	Leu	Leu	Ile	Asp
		595					600					605			
Leu	Cys	Ile	Leu	Ile	Cys	Trp	Gln	Ala	Val	Asp	Pro	Leu	Arg	Arg	Thr
	610					615					620				
Val	Glu	Lys	Tyr	Ser	Met	Glu	Pro	Asp	Pro	Ala	Gly	Arg	Asp	Ile	Ser
625					630					635					640
Ile	Arg	Pro	Leu	Leu	Glu	His	Cys	Glu	Asn	Thr	His	Met	Thr	Ile	Trp
				645					650					655	
Leu	Gly	Ile	Val	Tyr	Ala	Tyr	Lys	Gly	Leu	Leu	Met	Leu	Phe	Gly	Cys
			660					665						670	
Phe	Leu	Ala	Trp	Glu	Thr	Arg	Asn	Val	Ser	Ile	Pro	Ala	Leu	Asn	Asp
		675					680					685			
Ser	Lys	Tyr	Ile	Gly	Met	Ser	Val	Tyr	Asn	Val	Gly	Ile	Met	Cys	Ile
		690				695					700				
Ile	Gly	Ala	Ala	Val	Ser	Phe	Leu	Thr	Arg	Asp	Gln	Pro	Asn	Val	Gln
705					710					715					720
Phe	Cys	Ile	Val	Ala	Leu	Val	Ile	Ile	Phe	Cys	Ser	Thr	Ile	Thr	Leu
				725					730					735	
Cys	Leu	Val	Phe	Val	Pro	Lys	Leu	Ile	Thr	Leu	Arg	Thr	Asn	Pro	Asp
			740					745					750		
Ala	Ala	Thr	Gln	Asn	Arg	Arg	Phe	Gln	Phe	Thr	Gln	Asn	Gln	Lys	Lys
			755				760					765			
Glu	Asp	Ser	Lys	Thr	Ser	Thr	Ser	Val	Thr	Ser	Val	Asn	Gln	Ala	Ser
						775					780				
Thr	Ser	Arg	Leu	Glu	Gly	Leu	Gln	Ser	Glu	Asn	His	Arg	Leu	Arg	Met
785					790					795					800
Lys	Ile	Thr	Glu	Leu	Asp	Lys	Asp	Leu	Glu	Glu	Val	Thr	Met	Gln	Leu
				805					810					815	
Gln	Asp	Thr	Pro	Glu	Lys	Thr	Thr	Tyr	Ile	Lys	Gln	Asn	His	Tyr	Gln
			820					825					830		
Glu	Leu	Asn	Asp	Ile	Leu	Asn	Leu	Gly	Asn	Phe	Thr	Glu	Ser	Thr	Asp
		835					840					845			
Gly	Gly	Lys	Ala	Ile	Leu	Lys	Asn	His	Leu	Asp	Gln	Asn	Pro	Gln	Leu

850					855					860						
Gln 865	Trp	Asn	Thr	Thr	Glu 870	Pro	Ser	Arg	Thr	Cys 875	Lys	Asp	Pro	Ile	Glu 880	
Asp	Ile	Asn	Ser	Pro	Glu 885	His	Ile	Gln	Arg 890	Arg	Leu	Ser	Leu	Gln 895	Leu	
Pro	Ile	Leu	His 900	His	Ala	Tyr	Leu	Pro 905	Ser	Ile	Gly	Gly	Val 910	Asp	Ala	
Ser	Cys	Val 915	Ser	Pro	Cys	Val	Ser 920	Pro	Thr	Ala	Ser	Pro 925	Arg	His	Arg	
His 930	Val	Pro	Pro	Ser	Phe 935	Arg	Val	Met	Val	Ser	Gly 940	Leu				

What is claimed is:

1. An isolated nucleic acid encoding a GABA_BR2 polypeptide.
2. The nucleic acid of claim 1, wherein the nucleic acid is DNA.
3. The DNA of claim 2, wherein the DNA is cDNA.
4. The DNA of claim 2, wherein the DNA is genomic DNA.
5. The nucleic acid of claim 1, wherein the nucleic acid is RNA.
6. The nucleic acid of claim 1, wherein the nucleic acid encodes a mammalian GABA_BR2 polypeptide.
7. The nucleic acid of claim 1, wherein the nucleic acid encodes a rat GABA_BR2 polypeptide.
8. The nucleic acid of claim 1, wherein the nucleic acid encodes a human GABA_BR2 polypeptide.

9. The nucleic acid of claim 6, wherein the nucleic acid encodes a polypeptide characterized by an amino acid sequence in the transmembrane regions which has an identity of 90% or higher to the amino acid sequence in the transmembrane regions of the human GABA_BR2 polypeptide shown in Figures 5A-5D.
10. The nucleic acid of claim 6, wherein the nucleic acid encodes a mammalian GABA_BR2 polypeptide which has substantially the same amino acid sequence as does the GABA_BR2 polypeptide encoded by the plasmid BO-55 (ATCC Accession No. 209104).
11. The nucleic acid of claim 7, wherein the nucleic acid encodes a rat GABA_BR2 polypeptide which has an amino acid sequence encoded by the plasmid BO-55 (ATCC Accession No. 209104).
12. The nucleic acid of claim 7, wherein the nucleic acid encodes a rat GABA_BR2 polypeptide having substantially the same amino acid sequence as the amino acid sequence shown in Figures 4A-4D (Seq. ID No. 4).
13. The nucleic acid of claim 7, wherein the rat GABA_BR2 polypeptide has an amino acid sequence which comprises the amino acid sequence shown in Figures 4A-4D (Seq. ID No. 4).
14. The nucleic acid of claim 6, wherein the nucleic

acid encodes a mammalian GABA_BR2 polypeptide which has substantially the same amino acid sequence as does the GABA_BR2 polypeptide encoded by the plasmid pEXJT3T7-hGABAB2 (ATCC Accession No.).

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15. The nucleic acid of claim 8, wherein the human GABA_BR2 polypeptide comprises an amino acid sequence substantially the same as the amino acid sequence encoded by plasmid pEXJT3T7-hGABAB2 (ATCC Accession No.).

10

16. The nucleic acid of claim 8, wherein the human GABA_BR2 polypeptide comprises an amino acid sequence substantially the same as the amino acid sequence in Figures 23A-23D (Seq. ID No. 47).

15

17. The nucleic acid of claim 8, wherein the human GABA_BR2 polypeptide has an amino acid sequence which comprises the sequence shown in Figures 23A-23D (Seq. ID No. 47).

20

18. A purified GABA_BR2 protein.

19. A vector comprising the nucleic acid of claim 1.

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20. A vector comprising the nucleic acid of claim 8.

21. A vector of claim 19 adapted for expression in a

bacterial cell which comprises the regulatory elements necessary for expression of the nucleic acid in the bacterial cell operatively linked to the nucleic acid encoding a GABA_BR2 polypeptide so as to permit expression thereof.

5

22. A vector of claim 19 adapted for expression in an amphibian cell which comprises the regulatory elements necessary for expression of the nucleic acid in the amphibian cell operatively linked to the nucleic acid encoding a GABA_BR2 polypeptide so as to permit expression thereof.

10

23. A vector of claim 19 adapted for expression in a yeast cell which comprises the regulatory elements necessary for expression of the nucleic acid in the yeast cell operatively linked to the nucleic acid encoding a GABA_BR2 polypeptide so as to permit expression thereof.

15

24. A vector of claim 19 adapted for expression in an insect cell which comprises the regulatory elements necessary for expression of the nucleic acid in the insect cell operatively linked to the nucleic acid encoding the GABA_BR2 polypeptide so as to permit expression thereof.

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25. A vector of claim 24 which is a baculovirus.

30

26. A vector of claim 19 adapted for expression in a mammalian cell which comprises the regulatory

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elements necessary for expression of the nucleic acid in the mammalian cell operatively linked to the nucleic acid encoding a GABA_BR2 polypeptide so as to permit expression thereof.

5

27. A vector of claim 19 wherein the vector is a plasmid.

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28. The plasmid of claim 27 designated BO-55 (ATCC Accession No. 209104).

29. The plasmid of claim 27 designated pEXJT3T7-hGABAB2 (ATCC Accession No.).

15

30. A method of detecting a nucleic acid encoding a GABA_BR2 polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the GABA_BR2 polypeptide, wherein the probe has a unique sequence, which sequence is present within one of the two strands of the nucleic acid encoding the GABA_BR2 polypeptide contained in plasmid BO-55, and detecting hybridization of the probe to the nucleic acid.

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31. A method of detecting a nucleic acid encoding a GABA_BR2 polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically

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hybridizes with the nucleic acid encoding the GABA_BR2 polypeptide, wherein the probe has a unique sequence, which sequence is present within (a) the nucleic acid sequence shown in Figures 22A-22D (Seq. ID No. 46) or (b) the reverse complement to the nucleic acid sequence shown in Figures 22A-22D (Seq. ID No. 46), and detecting hybridization of the probe to the nucleic acid.

32. A method of detecting a nucleic acid encoding a GABA_BR2 polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the GABA_BR2 polypeptide, wherein the probe has a unique sequence, which sequence is present within one of the two strands of the nucleic acid encoding the GABA_BR2 polypeptide contained in plasmid pEXJT3T7-hGABAB2, and detecting hybridization of the probe to the nucleic acid.

33. A method of detecting a nucleic acid encoding a GABA_BR2 polypeptide, which comprises contacting the nucleic acid with a probe comprising at least 15 nucleotides, which probe specifically hybridizes with the nucleic acid encoding the GABA_BR2 polypeptide, wherein the probe has a unique sequence, which sequence is present within (a) the nucleic acid sequence shown in Figures 3A-3D (Seq. ID No. 3) or (b) the reverse complement to the nucleic acid sequence shown in Figures 3A-3D (Seq. ID No. 3), and detecting hybridization of the probe to the nucleic acid.

34. The method of any one of claims 30 to 33, wherein the nucleic acid is DNA.

5 35. The method of any one of claims 30 to 33, wherein the nucleic acid is RNA.

10 36. The method of any one of claims 30 to 33, wherein the probe comprises at least 15 nucleotides complementary to a unique segment of the sequence of the nucleic acid molecule encoding the GABA_BR2 polypeptide.

15 37. A method of detecting a nucleic acid encoding a GABA_BR2 polypeptide, which comprises contacting the nucleic acid with a probe comprising a nucleic acid of at least 15 nucleotides which is complementary to the antisense sequence of a unique segment of the sequence of the nucleic acid encoding the GABA_BR2 polypeptide, and
20 detecting hybridization of the probe to the nucleic acid.

25 38. A method of inhibiting translation of mRNA encoding a GABA_BR2 polypeptide which comprises contacting such mRNA with an antisense oligonucleotide having a sequence capable of specifically hybridizing to the mRNA of claim 5, so as to prevent translation of the mRNA.

30 39. A method of inhibiting translation of mRNA

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46. A method of claim 38 or 39, wherein the oligonucleotide is coupled to a substance which inactivates mRNA.

5 47. A method of claim 46, wherein the substance which inactivates mRNA is a ribozyme.

10 48. A pharmaceutical composition which comprises an amount of the antibody of claim 41 effective to block binding of a ligand to the GABA_BR2 polypeptide and a pharmaceutically acceptable carrier.

15 49. A transgenic, nonhuman mammal expressing DNA encoding a GABA_BR2 polypeptide of claim 1.

20 50. A transgenic, nonhuman mammal comprising a homologous recombination knockout of the native GABA_BR2 polypeptide.

25 51. A transgenic, nonhuman mammal whose genome comprises antisense DNA complementary to DNA encoding a GABA_BR2 polypeptide of claim 1 so placed as to be transcribed into antisense mRNA which is complementary to mRNA encoding such GABA_BR2 polypeptide and which hybridizes to such mRNA encoding such GABA_BR2 polypeptide, thereby reducing its translation.

52. The transgenic, nonhuman mammal of claim 49 or 50, wherein the DNA encoding the GABA_BR2 polypeptide additionally comprises an inducible promoter.

5 53. The transgenic, nonhuman mammal of claim 49 or 50, wherein the DNA encoding the GABA_BR2 polypeptide additionally comprises tissue specific regulatory elements.

10 54. A transgenic, nonhuman mammal of any one of claims 49, 50 or 51, wherein the transgenic, nonhuman mammal is a mouse.

15 55. A method of detecting the presence of a GABA_BR2 polypeptide on the surface of a cell which comprises contacting the cell with the antibody of claim 41 under conditions permitting binding of the antibody to the polypeptide, detecting the presence of the antibody bound to the cell, and
20 thereby detecting the presence of a GABA_BR2 polypeptide on the surface of the cell.

56. A method of preparing the purified GABA_BR2 polypeptide of claim 18 which comprises:

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a. inducing cells to express a GABA_BR2 polypeptide;

b. recovering the polypeptide so expressed from

the induced cells; and

c. purifying the polypeptide so recovered.

5 57. A method of preparing the purified GABA_BR2 polypeptide of claim 18 which comprises:

a. inserting a nucleic acid encoding the GABA_BR2 polypeptide into a suitable vector;

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b. introducing the resulting vector in a suitable host cell;

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c. placing the resulting cell in suitable condition permitting the production of the GABA_BR2 polypeptide;

d. recovering the polypeptide produced by the resulting cell; and

20

e. isolating or purifying the polypeptide so recovered.

25 58. A GABA_BR1/R2 receptor comprising two polypeptides, one of which is a GABA_BR2 polypeptide and another of which is a GABA_BR1 polypeptide.

59. A method of forming a GABA₈R1/R2 receptor which comprises inducing cells to express both a GABA₈R1 polypeptide and a GABA₈R2 polypeptide.
- 5 60. An antibody capable of binding to a GABA₈R1/R2 receptor, wherein the GABA₈R2 polypeptide is encoded by the nucleic acid of claim 1.
- 10 61. The antibody of claim 60, wherein the GABA₈R2 polypeptide is a human GABA₈R2 polypeptide.
62. An antibody capable of competitively inhibiting the binding of the antibody of claim 60 to a GABA₈R1/R2 receptor.
- 15 63. An antibody of claim 60, wherein the antibody is a monoclonal antibody.
- 20 64. A monoclonal antibody of claim 63 directed to an epitope of a GABA₈R1/R2 receptor present on the surface of a GABA₈R1/R2 polypeptide expressing cell.
- 25 65. A pharmaceutical composition which comprises an amount of the antibody of claim 60 effective to block binding of a ligand to the GABA₈R1/R2 receptor and a pharmaceutically acceptable carrier.

66. A transgenic, nonhuman mammal expressing a GABA_BR1/R2 receptor, which is not naturally expressed by the mammal.
- 5 67. A transgenic, nonhuman mammal comprising a homologous recombination knockout of the native GABA_BR1/R2 receptor.
- 10 68. A transgenic, nonhuman mammal of claim 66 or 67, wherein the transgenic nonhuman mammal is a mouse.
- 15 69. A method of detecting the presence of a GABA_BR1/R2 receptor on the surface of a cell which comprises contacting the cell with the antibody of claim 60 under conditions permitting binding of the antibody to the receptor, detecting the presence of the antibody bound to the cell, and thereby detecting the presence of a GABA_BR1/R2 receptor on the surface of the cell.
- 20 70. A method of determining the physiological effects of varying levels of activity of GABA_BR1/R2 receptors which comprises producing a transgenic nonhuman mammal of claim 66 whose levels of GABA_BR1/R2 receptor activity vary due to the presence of an inducible promoter which regulates GABA_BR1/R2 receptor expression.
- 25 71. A method of determining the physiological effects of varying levels of activity of GABA_BR1/R2 receptors which comprises producing a panel of
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transgenic nonhuman mammals of claim 66, each expressing a different amount of GABA_BR1/R2 receptor.

5 72. A method for identifying an antagonist capable of alleviating an abnormality, by decreasing the activity of a GABA_BR1/R2 receptor comprising administering a compound to the transgenic nonhuman mammal of claim 66 or 68, and determining
10 whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal, the alleviation of the abnormality identifying the compound as the antagonist.

15 73. An antagonist identified by the method of claim 72.

20 74. A pharmaceutical composition comprising an antagonist of claim 73 and a pharmaceutically acceptable carrier.

25 75. A method of treating an abnormality in a subject wherein the abnormality is alleviated by decreasing the activity of a GABA_BR1/R2 receptor which comprises administering to a subject an effective amount of the pharmaceutical composition of claim 74, thereby treating the abnormality.

30 76. A method for identifying an agonist capable of alleviating an abnormality, by increasing the

activity of a GABA_BR1/R2 receptor comprising administering a compound to the transgenic nonhuman mammal of claim 66 or 68, and determining whether the compound alleviates the physical and behavioral abnormalities displayed by the transgenic, nonhuman mammal, the alleviation of the abnormality identifying the compound as the agonist.

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77. An agonist identified by the method of claim 76.

78. A pharmaceutical composition comprising an agonist of claim 76 and a pharmaceutically acceptable carrier.

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79. A method for treating an abnormality in a subject wherein the abnormality is alleviated by increasing the activity of a GABA_BR1/R2 receptor which comprises administering to a subject an effective amount of the pharmaceutical composition of claim 78, thereby treating the abnormality.

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80. A cell which expresses on its surface a mammalian GABA_BR1/R2 receptor that is not naturally expressed on the surface of such cell.

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81. A cell of claim 80, wherein the mammalian GABA_BR1/R2 receptor comprises two polypeptides, one of which is a GABA_BR2 polypeptide and another of which is a GABA_BR1 polypeptide.

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82. A process for identifying a chemical compound which specifically binds to a GABA_BR1/R2 receptor which comprises contacting cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the GABA_BR1/R2 receptor.

83. A process for identifying a chemical compound which specifically binds to a GABA_BR1/R2 receptor which comprises contacting a membrane fraction from a cell extract of cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the compound under conditions suitable for binding, and detecting specific binding of the chemical compound to the GABA_BR1/R2 receptor.

84. The process of claim 82 or 83, wherein the GABA_BR1/R2 receptor is a mammalian GABA_BR1/R2 receptor.

85. The process of claim 82 or 83, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid BO-55 (ATCC Accession No. 209104).

86. The process of claim 82 or 83, wherein the

GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same sequence as the amino acid sequence shown in Figures 23A-23D (Seq. ID No. 47).

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87. The process of claim 82 or 83, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has the amino acid sequence shown in Figures 23A-23D (Seq. ID No. 47).

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88. The process of claims 82 or 83, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid pEXJT3T7-hGABAB2 (ATCC Accession No.).

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89. The process of claim 82 or 83, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as the sequence shown in Figures 23A-23D (Seq. ID No. 47).

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90. The process of claim 82 or 83, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has the sequence shown in Figures 23A-23D (Seq. ID No. 47).

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91. The process of claim 89, wherein the compound is not previously known to bind to a GABA_BR1/R2 receptor.

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92. A compound identified by the process of claim 91.

93. A process of claim 89, wherein the cell is an insect cell.

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94. A process of claim 89, wherein the cell is a mammalian cell.

95. A process of claim 94, wherein the cell is nonneuronal in origin.

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96. A process of claim 95, wherein the nonneuronal cell is a COS-7 cell, 293 human embryonic kidney cell, a CHO cell, a NIH-3T3 cell a mouse Y1 cell or LM(tk-) cell.

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97. A process of claim 94, wherein the compound is not previously known to bind to a GABA_BR1/R2 receptor.

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98. A compound identified by the process of claim 97.

99. A process involving competitive binding for identifying a chemical compound which specifically binds to a GABA_BR1/R2 receptor which comprises separately contacting cells expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with both the chemical compound and a

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second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the GABA_BR1/R2 receptor, a decrease in the binding of the second chemical compound to the GABA_BR1/R2 receptor in the presence of the chemical compound indicating that the chemical compound binds to the GABA_BR1/R2 receptor.

100. A process involving competitive binding for identifying a chemical compound which specifically binds to a human GABA_BR1/R2 receptor which comprises separately contacting a membrane fraction from a cell extract of cells expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with both the chemical compound and a second chemical compound known to bind to the receptor, and with only the second chemical compound, under conditions suitable for binding of both compounds, and detecting specific binding of the chemical compound to the GABA_BR1/R2 receptor, a decrease in the binding of the second chemical compound to the GABA_BR1/R2 receptor in the presence of the chemical compound indicating that the chemical compound binds to the GABA_BR1/R2 receptor.

101. A process of claim 99 or 100, wherein the GABA_BR1/R2 receptor is a mammalian GABA_BR1/R2 receptor.

102. The process of claim 101, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that encoded by plasmid BO-55 (ATCC Accession No. 209104).

103. The process of claim 99 or 100, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that shown in Figures 23A-23D (Seq. ID No. 47).

104. The process of claim 99 or 100, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has the amino acid sequence shown in Figures 23A-23D (Seq. ID No. 47).

105. The process of claim 99 or 100, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that encoded by plasmid pEXJT3T7-hGABAB2 (ATCC Accession No.).

106. The process of claim 99 or 100, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as the sequence shown in Figures 23A-23D (Seq. ID No. 47).

107. The process of claim 99 or 100, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide

which has the sequence shown in Figures 23A-23D
(Seq. ID No. 47).

5 108. The process of claim 107, wherein the cell is an
insect cell.

109. The process of claim 107, wherein the cell is a
mammalian cell.

10 110. The process of claim 109, wherein the cell is
nonneuronal in origin.

15 111. The process of claim 110, wherein the nonneuronal
cell is a COS-7 cell, 293 human embryonic kidney
cell, a CHO cell, a NIH-3T3 cell a mouse Y1 cell
or LM(tk-) cell.

20 112. The process of claim 109, wherein the compound is
not previously known to bind to a GABA_BR1/R2
receptor.

113. A compound identified by the process of claim 112.

25 114. A method of screening a plurality of chemical
compounds not known to bind to a GABA_BR1/R2
receptor to identify a compound which specifically
binds to the GABA_BR1/R2 receptor, which comprises

(a) contacting cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with a compound known to bind specifically to the GABA_BR1/R2 receptor;

(b) contacting the same cells as in step (a) with the plurality of compounds not known to bind specifically to the GABA_BR1/R2 receptor, under conditions permitting binding of compounds known to bind the GABA_BR1/R2 receptor;

(c) determining whether the binding of the compound known to bind specifically to the GABA_BR1/R2 receptor is reduced in the presence of the plurality of the compounds, relative to the binding of the compound in the absence of the plurality of compounds, and if the binding is reduced;

(d) separately determining the extent of binding to the GABA_BR1/R2 receptor of each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which specifically binds to the GABA_BR1/R2 receptor.

115. A method of screening a plurality of chemical compounds not known to bind to a GABA_BR1/R2 receptor to identify a compound which specifically binds to the GABA_BR1/R2 receptor, which comprises

(a) contacting a membrane fraction extract from cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with a compound known to bind specifically to the GABA_BR1/R2 receptor;

(b) contacting the same membrane fraction as in step (a) with the plurality of compounds not known to bind specifically to the GABA_BR1/R2 receptor, under conditions permitting binding of compounds known to bind the GABA_BR1/R2 receptor;

(c) determining whether the binding of the compound known to bind specifically to the GABA_BR1/R2 receptor is reduced in the presence of the plurality of compounds, relative to the binding of the compound in the absence of the plurality of compounds, and if the binding is reduced;

(d) separately determining the extent of binding to the GABA_BR1/R2 receptor of each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which specifically binds to the GABA_BR1/R2 receptor.

116. A method of claim 114 or 115, wherein the GABA_BR1/R2 receptor is a mammalian GABA_BR1/R2

receptor.

117. A method of either of claim 114 or 115, wherein the cell is a mammalian cell.

5

118. A method of claim 117, wherein the mammalian cell is non-neuronal in origin.

119. The method of claim 118, wherein the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell, a CHO cell, a mouse Y1 cell or an NIH-3T3 cell.

10

120. A process for determining whether a chemical compound is a GABA_BR1/R2 receptor agonist which comprises contacting cells with the compound under conditions permitting the activation of the GABA_BR1/R2 receptor, and detecting an increase in GABA_BR1/R2 receptor activity, so as to thereby determine whether the compound is a GABA_BR1/R2 receptor agonist.

15

20

121. A process for determining whether a chemical compound is a GABA_BR1/R2 receptor antagonist which comprises contacting cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the compound in the presence of a known GABA_BR1/R2 receptor agonist, under conditions permitting the activation of the GABA_BR1/R2 receptor, and

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detecting a decrease in GABA_BR1/R2 receptor activity, so as to thereby determine whether the compound is a GABA_BR1/R2 receptor antagonist.

- 5 122. A process of claim 120 or 121, wherein the cells additionally express nucleic acid encoding GIRK1 and GIRK4.
- 10 123. A process of any one of claims 120, 121, or 122, wherein the GABA_BR2 receptor is a mammalian GABA_BR2 receptor.
- 15 124. A pharmaceutical composition which comprises an amount of a GABA_BR1/R2 receptor agonist determined to be an agonist by the process of claim 120 effective to increase activity of a GABA_BR1/R2 receptor and a pharmaceutically acceptable carrier.
- 20 125. A pharmaceutical composition of claim 124, wherein the GABA_BR1/R2 receptor agonist was not previously known.
- 25 126. A pharmaceutical composition which comprises an amount of a GABA_BR1/R2 receptor antagonist determined to be an antagonist the process of claim 121 effective to reduce activity of a GABA_BR1/R2 receptor and a pharmaceutically acceptable carrier.

127. A pharmaceutical composition of claim 126, wherein the GABA_BR1/R2 receptor antagonist was not previously known.

5 128. A process for determining whether a chemical compound activates a GABA_BR1/R2 receptor, which comprises contacting cells producing a second messenger response and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells
10 do not normally express the GABA_BR1/R2 receptor, with the chemical compound under conditions suitable for activation of the GABA_BR1/R2 receptor, and measuring the second messenger response in the presence and in the absence of the chemical
15 compound, a change in the second messenger response in the presence of the chemical compound indicating that the compound activates the GABA_BR1/R2 receptor.

20 129. The process of claim 128, wherein the second messenger response comprises potassium channel activation and the change in second messenger is an increase in the level of potassium current.

25 130. A process for determining whether a chemical compound inhibits activation of a GABA_BR1/R2 receptor, which comprises separately contacting cells producing a second messenger response and expressing on their cell surface the GABA_BR1/R2
30 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with both the chemical compound and a second chemical compound known to activate the GABA_BR1/R2 receptor, and with

only the second chemical compound, under conditions suitable for activation of the GABA_BR1/R2 receptor, and measuring the second messenger response in the presence of only the second chemical compound and in the presence of both the second chemical compound and the chemical compound, a smaller change in the second messenger response in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound indicating that the chemical compound inhibits activation of the GABA_BR1/R2 receptor.

131. The process of claim 130, wherein the second messenger response comprises potassium channel activation and the change in second messenger response is a smaller increase in the level of inward potassium current in the presence of both the chemical compound and the second chemical compound than in the presence of only the second chemical compound.

132. A process of any one of claims 128, 129, 130 or 131, wherein the GABA_BR1/R2 receptor is a mammalian GABA_BR1/R2 receptor.

133. The process of claim 132, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid BO-55 (ATCC Accession No. 209104).

134. The process of claim 132, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that shown in Figures 4A-4D (Seq. ID No. 4).

5

135. The process of claim 132, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that shown in Figures 23A-23D (Seq. ID No. 47).

10

136. The process of claim 132, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has the sequence, shown in Figures 23A-23D (Seq. ID No. 47).

15

137. The process of claim 132, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid pEXJT3T7-hGABAB2 (ATCC Accession No.).

20

138. The process of any one of claims 128-131, wherein the cell is an insect cell.

25

139. The process of any one of claims 128-131, wherein the cell is a mammalian cell.

140. The process of claim 139, wherein the mammalian cell is nonneuronal in origin.

141. The process of claim 140, wherein the nonneuronal cell is a COS-7 cell, CHO cell, 293 human embryonic kidney cell, NIH-3T3 cell or LM(tk-) cell.

5

142. The process of claim 139, wherein the compound was not previously known to activate or inhibit a GABA_BR1/R2 receptor.

10

143. A compound determined by the process of claim 142.

15

144. A pharmaceutical composition which comprises an amount of a GABA_BR1/R2 receptor agonist determined by the process of claim 128 or 129 effective to increase activity of a GABA_BR1/R2 receptor and a pharmaceutically acceptable carrier.

20

145. A pharmaceutical composition of claim 144, wherein the GABA_BR1/R2 receptor agonist was not previously known.

25

146. A pharmaceutical composition which comprises an amount of a GABA_BR1/R2 receptor antagonist determined by the process of claim 130 or 131 effective to reduce activity of a GABA_BR1/R2 receptor and a pharmaceutically acceptable carrier.

147. A pharmaceutical composition of claim 146, wherein

the GABA_BR1/R2 receptor antagonist was not previously known.

148. A method of screening a plurality of chemical compounds not known to activate a GABA_BR1/R2 receptor to identify a compound which activates the GABA_BR1/R2 receptor which comprises:

(a) contacting cells containing nucleic acid encoding and expressing on their cell surface the GABA_BR1/R2 receptor, wherein such cells do not normally express the GABA_BR1/R2 receptor, with the plurality of compounds not known to activate the GABA_BR1/R2 receptor, under conditions permitting activation of the GABA_BR1/R2 receptor;

(b) determining whether the activity of the GABA_BR1/R2 receptor is increased in the presence of the compounds, and if it is increased;

(c) separately determining whether the activation of the GABA_BR1/R2 receptor is increased by each compound included in the plurality of compounds, so as to thereby identify the compound or compounds present in such plurality of compounds which activates the GABA_BR1/R2 receptor.

149. The process of claim 148, wherein the cells

express nucleic acid encoding GIRK1 and GIRK4.

150. A method of claim 148 or 149, wherein the
GABA_BR1/R2 receptor is a mammalian GABA_BR1/R2
receptor.

151. A method of screening a plurality of chemical
compounds not known to inhibit the activation of a
GABA_BR1/R2 receptor to identify a compound which
inhibits the activation of the GABA_BR1/R2 receptor,
which comprises:

- (a) contacting cells containing nucleic acid
encoding and expressing on their cell surface
the GABA_BR1/R2 receptor, wherein such cells do
not normally express the GABA_BR1/R2 receptor,
with the plurality of compounds in the
presence of a known GABA_BR1/R2 receptor
agonist, under conditions permitting
activation of the GABA_BR1/R2 receptor;
- (b) determining whether the activation of the
GABA_BR1/R2 receptor is reduced in the presence
of the plurality of compounds, relative to
the activation of the GABA_BR1/R2 receptor in
the absence of the plurality of compounds,
and if it is reduced;
- (c) separately determining the inhibition of
activation of the GABA_BR1/R2 receptor for each
compound included in the plurality of

compounds, so as to thereby identify the compound or compounds present in such a plurality of compounds which inhibits the activation of the GABA_BR1/R2 receptor.

5

152. The process of claim 151, wherein the cells express nucleic acid encoding GIRK1 and GIRK4.

10

153. A method of claim 151 or 152, wherein the GABA_BR1/R2 receptor is a mammalian GABA_BR1/R2 receptor.

15

154. A method of any one of claims 148, 149, 151, or 152, wherein the cell is a mammalian cell.

20

155. A method of claim 154, wherein the mammalian cell is non-neuronal in origin.

25

156. The method of claim 155, wherein the non-neuronal cell is a COS-7 cell, a 293 human embryonic kidney cell, a LM(tk-) cell or an NIH-3T3 cell.

157. A pharmaceutical composition comprising a compound identified by the method of claim 148 or 149, effective to increase GABA_BR1/R2 receptor activity and a pharmaceutically acceptable carrier.

158. A pharmaceutical composition comprising a compound

identified by the method of claim 151 or 152,
effective to decrease GABA_BR1/R2 receptor activity
and a pharmaceutically acceptable carrier.

5 159. A process for determining whether a chemical
compound is a GABA_BR1/R2 receptor agonist, which
comprises preparing a membrane fraction from cells
which comprise nucleic acid encoding and
expressing on their cell surface the GABA_BR1/R2
10 receptor, wherein such cells do not normally
express the GABA_BR1/R2 receptor, separately
contacting the membrane fraction with both the
chemical compound and GTPγS, and with only GTPγS,
under conditions permitting the activation of the
15 GABA_BR1/R2 receptor, and detecting GTPγS binding to
the membrane fraction, an increase in GTPγS
binding in the presence of the compound indicating
that the chemical compound activates the GABA_BR1/R2
receptor.

20
160. A process for determining whether a chemical
compound is a GABA_BR1/R2 receptor antagonist, which
comprises preparing a membrane fraction from cells
which comprise nucleic acid encoding and
25 expressing on their cell surface the GABA_BR1/R2
receptor, wherein such cells do not normally
express the GABA_BR1/R2 receptor, separately
contacting the membrane fraction with the chemical
compound, GTPγS and a second chemical compound
30 known to activate the GABA_BR1/R2 receptor, with
GTPγS and only the second compound, and with GTPγS
alone, under conditions permitting the activation
of the GABA_BR1/R2 receptor, detecting GTPγS binding
to each membrane fraction, and comparing the
35 increase in GTPγS binding in the presence of the

compound and the second compound relative to the binding of GTP γ S alone, to the increase in GTP γ S binding in the presence of the second chemical compound known to activate the GABA $_B$ R1/R2 receptor relative to the binding of GTP γ S alone, a smaller increase in GTP γ S binding in the presence of the compound and the second compound indicating that the compound is a GABA $_B$ R1/R2 receptor antagonist.

161. A process of claim 159 or 160, wherein the GABA $_B$ R2 receptor is a mammalian GABA $_B$ R2 receptor.

162. The process of claim 161, wherein the GABA $_B$ R1/R2 receptor comprises a GABA $_B$ R2 polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid BO-55 (ATCC Accession No. 209104).

163. The process of claim 162, wherein the GABA $_B$ R1/R2 receptor comprises a GABA $_B$ R2 polypeptide which has substantially the same amino acid sequence as that shown in Figures 4A-4D (Seq. ID No. 4).

164. The process of claim 161, wherein the GABA $_B$ R1/R2 receptor comprises a GABA $_B$ R2 polypeptide which has substantially the same amino acid sequence as that encoded by the plasmid pEXJT3T7-hGABAB2 (ATCC Accession No.).

165. The process of claim 161, wherein the GABA $_B$ R1/R2 receptor comprises a GABA $_B$ R2 polypeptide which has

substantially the same amino acid sequence as that shown in Figures 23A-23D (Seq. ID No. 47).

5 166. The process of claim 161, wherein the GABA_BR1/R2 receptor comprises a GABA_BR2 polypeptide which has the sequence shown in Figures 23A-23D (Seq. ID No. 47).

10 167. The process of claim 159 or 160, wherein the cell is an insect cell.

168. The process of claim 159 or 160, wherein the cell is a mammalian cell.

15 169. The process of claim 168, wherein the mammalian cell is nonneuronal in origin.

20 170. The process of claim 169, wherein the nonneuronal cell is a COS-7 cell, CHO cell, 293 human embryonic kidney cell, NIH-3T3 cell or LM(tk-) cell.

25 171. The process of claim 170, wherein the compound was not previously known to be an agonist or antagonist of a GABA_BR1/R2 receptor.

172. A compound determined to be an agonist or antagonist of a GABA_BR1/R2 receptor by the process

of claim 171.

5 173. A method of treating spasticity in a subject which comprises administering to the subject an amount of a compound which is an agonist of a GABA_BR1/R2 receptor effective to treat spasticity in the subject.

10 174. A method of treating asthma in a subject which comprises administering to the subject an amount of a compound which is a GABA_BR1/R2 receptor agonist effective to treat asthma in the subject.

15 175. A method of treating incontinence in a subject which comprises administering to the subject an amount of a compound which is a GABA_BR1/R2 receptor agonist effective to treat incontinence in the subject.

20 176. A method of decreasing nociception in a subject which comprises administering to the subject an amount of a compound which is a GABA_BR1/R2 receptor agonist effective to decrease nociception in the subject.

25 177. A use of a GABA_BR2 agonist as an antitussive agent which comprises administering to the subject an amount of a compound which is a GABA_BR1/R2 receptor agonist effective as an antitussive agent in the
30 subject.

178. A method of treating drug addiction in a subject which comprises administering to the subject an amount of a compound which is a GABA_BR1/R2 receptor agonist effective to treat drug addiction in the subject.

5

179. A method of treating Alzheimer's disease in a subject which comprises administering to the subject an amount of a compound which is a GABA_BR1/R2 receptor antagonist effective to treat Alzheimer's disease in the subject.

10

182. A process for making a composition of matter which specifically binds to a GABA_BR1/R2 receptor which comprises identifying a chemical compound using the process of any of claims, 82, 83, 99, 100, 114 or 115 and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof.

15

20

183. A process for making a composition of matter which specifically binds to a GABA_BR1/R2 receptor which comprises identifying a chemical compound using the process of any of claims 120, 128, or 148 and then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof.

25

184. A process for making a composition of matter which specifically binds to a GABA_BR1/R2 receptor which comprises identifying a chemical compound using the process of any of claims 121, 130, or 151 and

30

then synthesizing the chemical compound or a novel structural and functional analog or homolog thereof.

- 5 185. The process of any of claims 182, 183, or 184,
 wherein the GABA_BR1/R2 receptor is a human
 GABA_BR1/R2 receptor.
- 10 186. A process for preparing a pharmaceutical
 composition which comprises admixing a
 pharmaceutically acceptable carrier and a
 pharmaceutically acceptable amount of a chemical
 compound identified by the process of any of
15 claims 82, 83, 99, 100, 114 or 115 or a novel
 structural and functional analog or homolog
 thereof.
- 20 187. A process for preparing a pharmaceutical
 composition which comprises admixing a
 pharmaceutically acceptable carrier and a
 pharmaceutically acceptable amount of a chemical
 compound identified by the process of any of
 claims 120, 128, or 148 or a novel structural and
 functional analog or homolog thereof.
- 25 188. A process for preparing a pharmaceutical
 composition which comprises admixing a
 pharmaceutically acceptable carrier and a
 pharmaceutically acceptable amount of a chemical
30 compound identified by the process of any of
 claims 121, 130, or 151 or a novel structural and
 functional analog or homolog thereof.

189. The process of any of claims 186, 187, or 188, wherein the GABA_bR1/R2 receptor is a human GABA_bR1/R2. receptor.

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DNA ENCODING A GABA_BR2 POLYPEPTIDE AND USES THEREOF

ABSTRACT OF THE DISCLOSURE

5 This invention provides isolated nucleic acids encoding
a mammalian GABA_BR2 polypeptide, an isolated GABA_BR2
protein, vectors comprising isolated nucleic acid
encoding mammalian GABA_BR2 polypeptides, cells
expressing mammalian GABA_BR1/R2 receptors, antibodies
10 directed to an epitope on mammalian GABA_BR2 polypeptides
or mammalian GABA_BR1/R2 receptors, nucleic acid probes
useful for detecting nucleic acids encoding mammalian
GABA_BR2 polypeptides, antisense oligonucleotides
complementary to unique sequences of nucleic acids
15 encoding mammalian GABA_BR2 polypeptides, nonhuman
transgenic animals which express DNA encoding normal or
mutant mammalian GABA_BR1/R2 receptors, as well as
methods of screening compounds acting as agonists or
antagonists of mammalian GABA_BR1/R2 receptors.

FIGURE 1A

-243	TGACCTCGGGCAGGTCCTGGTGACAGAGCGTCGCCAAGGACGCCGAGAGGGAGGCGGGGAT	-184
-183	TGCCCAGACATCCTTCAGCGAAGTGCAATGTGTGTTGTAAACCATCGTTGGCTGTTCGGGA	-124
-123	GACCGCGAGGACCGGTCCAGGCTCGGGCGAGTCGAGGGCGAGGAGGAGCGCGGTGAGT	-64
-63	GAGCAGAGTCCAGAGCCGTGCGCCCCCAGAACTGCCGCTCCGCCCTGCACCCCCCGCGC	-4
-3	GCCATGCCCAGTTGCCCCCCGCGCTCTGCTACGGGCCCGCTCTCCATCATGGGCCCTCATG	57
58	CCGCTCACCAAGAGGTGGCCAAAGGCAGCATCGGGCGGCTGTGCTCCCCGCCGTGGAA	117
118	CTGGCCATCGAGCAGATCCGCAACGAGTCACTCCTGCGCCCCCTACTTCCTCGACCTGCGG	177
178	CTCTATGACACGGAGTGCACACGCAAAAGGGTTGAAAGCCTTCTACGATCGGATAAAA	237
238	TACGGGCCGAACCACTTGATGGTGTTTGGAGGCGTCTGTCCATCCGTCACATCCATCAT	297
298	GCAGAGTCCCTCCAAGGCTGGAAATCTGGTGCAGCTTTCCTTTGCTGCAACACGCCCTGTT	357
358	CTAGCCGATAAGAAAAAATACCCCTTATTTCTTTCGGACCGTCCCATCAGACAAATGCGGTG	417
418	AATCCAGCCATTCTGAAGTTGCTCAAGCACTACCAGTGAAGCGGTGGGCACGCTGACG	477
478	CAAGACGTTTCAGAGGTTCTCTGAGGTGCGGAATGACCTGACTGGAGTTCTGTATGGCGAG	537

FIGURE 1B

538 GACATTGAGATTTCAGACACCGAGAGCTTCTCCAACGATCCCTGTACCAGTGTCAAAAAG 597
598 CTGAAGGGGAATGATGTGCGGATCATCCTTGGCCAGTTTGACCAGAAATATGGCAGCAAAA 657
658 GTGTTCTGTTGCATACGAGGAGAACATGTATGTTAGTAAATATCAGTGGATCATTTCCG 717
718 GGC TGGTACGAGCCTTCTTGGTGGGAGCAGGTGCCACGGAAGCCAACTCATCCCGCTGC 777
778 CTCCGGAAGAACTGCTTGTCTGCCATGGAGGGCTACATTGGCGTGGATTTCGAGCCCCCTG 837
838 AGCTCCAAGCAGATCAAGACCATCTCAGGAAGACTCCACAGCAGTATGAGAGAGAGTAC 897
898 AACAAACAGCGGTCAGGCGTGGGGCCAGCAAGTTCCACGGGTACGCCACGAGCGGCATC 957
958 TGGGTCA TCGCCAAGACACTGCAGAGGGCCATGGAGACACTGCATGCCAGCAGCCGGCAC 1017
1018 CAGCGGATCCAGGACTTCAACTACCGGACCAACACGCTGGGCAGGATCATCCTCAATGCC 1077
1078 ATGAACGAGACCAACTTCTTCGGGGTCACGGGTCAAGTTGTATTCCGGAATGGGAGAGA 1137
1138 ATGGGGACCATTAATTACTCAATTTCAAGACAGCAGGAGGTGAAGGTGGGAGAGTAC 1197
1198 AACGCTGTGGCCGACACACTGGAGATCATCAATGACACCATCAGGTTCCAAGGATCCGAA 1257
1258 CCACCAAAGACAAGACCATCATCCTGGAGCAGCTGCGGAAGATCTCCCTACCTCTCTAC 1317

FIGURE 1C

1318 AGCATCCTCTGTGCCCCACCATCCTCGGGATGATCATGGCCAGTGCTTTTCTCTTCTTC 1377

1378 AACATCAAGAACCGGAATCAGAAAGCTCATAAAGATGTCGAGTCCATACATGAACAACCTT 1437

1438 ATCATCCTTGGAGGGATGCTTTCCTATGCTTCCATATTTCTCTTTGGCCCTTGATGGATCC 1497

1498 TTTGTCTCTGAAAAGACCTTTGAAACACTTTGCACCCGTCAGGACCTGGATTCTCACCCGTG 1557

1558 GGCTACACGACCGCTTTTGGGGCCATGTTTGCAAGACCTGGAGAGTCCACGCCATCTTC 1617

1618 AAAAATGTGAAAATGAAGAAGAAGATCATCAAGGACCAGAAACTGCTTGTGATCGTGGGG 1677

1678 GGCAATGCTGCTGATCGACCTGTGTATCCTGATCTGCTGGCAGGCTGTGGACCCCTGCCGA 1737

1738 AGGACAGTGGAGAAAGTACAGCATGGAGCCGGACCCAGCAGGACGGGATATCTCCATCCGC 1797

1798 CCTCTCCTGGAGCACCTGTGAGAACACCCATATGACCATCTGGCTTGGCATCGTCTATGCC 1857

1858 TACAAGGGACTTCTCATGTTGTTCCGGTTGTTTCTTAGCTTGGGAGACCCGCAACGTCAGC 1917

1918 ATCCCCGCACTCAACGACAGCAAGTACATCGGGATGAGTGTCTACAACGTGGGGATCATG 1977

1978 TGCATCATCGGGCCGCTGTCTCCTTCCCTGACCCGGGACCAGCCCAATGTGCAGTTCTGC 2037

2038 ATCGTGGCTCTGGTTCATCATCTTCTGCAGCACCATCACCCCTCTGCCCTGGTATTCGTGCCG 2097

FIGURE 1D

2098	AAGCTCATCACCCCTGAGAAACAACCCAGATGCAGCAACGCAGAAACAGCGGATTCAGTTC	2157
2158	ACTCAGAAATCAGAAAGAAAGATCTAAACGTCCACCTCGGTCAACAGTGTGAACCAA	2217
2218	GCCAGCACATCCCGCTGGAGGGCCTACAGTCAGAAACCATCGCCTGCGAATGAAGATC	2277
2278	ACAGAGCTGGATAAAGACTTTGGAAGAGGTCACCATGCAGCTGCAGGACACACAGAAAAG	2337
2338	ACCACCTACATTAAACAGAACCCACTACCAAGAGCTCAATGACATCCCTCAACCTGGGAAAC	2397
2398	TTCACTGAGAGCACAGATGGAGGAAAGGCCATTTTAAAAAATCACCTCGATCAAAAATCCC	2457
2458	CAGCTACAGTGGAACACACACAGAGCCCTCTCGAACATGCAAAAGATCCTATAGAAGATATA	2517
2518	AACTCTCCAGAACACATCCAGCGTCGGCTGTCCCTCCAGCTCCCCCATCTCCACACGCC	2577
2578	TACCTCCCATCCATCGGAGGCGTGGACGCCAGCTGTGTCAGCCCTCGGTCAGCCCCACC	2637
2638	GCCAGCCCCCGCCACAGACATGTGCCACCCCTCCTCCGAGTCATGGTCTCGGGCCTGTAA	2697
2698	GGTGGGAGGCCCTGGGCCCGGGCCCTCCCCCGTGACAGAAACACACTGGGCAGAGGGGTC	2757
2758	TGCTGCAGAAACACTGTTCGGCTCTGGCTGCGGAGAAAGCTGGGCACCATGGCTGGCCTCTC	2817
2818	AGGACCACTCGGATGGCACTCAGGTGGACAGGACGGGGCAGGGGAGACTTGGCACCTGA	2877

FIGURE 1E

2878	CCTCGAGCCCTTATTGTGAAGTCCTTATTTCCTCACAAAGAAGAGGAACGGAAATGGGAC	2937
2938	GTCTTCCCTTAACATCTGCAACAAGGAGGCGCTGGGATATCAAACTTGCAAAAAA	2997
2998	AAAA	3001

FIGURE 2A

1	M	P	S	C	P	A	R	S	A	T	G	P	L	S	I	M	G	L	M	P	20
21	L	T	K	E	V	A	K	G	S	I	G	R	G	V	L	P	A	V	E	L	40
41	A	I	E	Q	I	R	N	E	S	L	L	R	P	Y	F	L	D	L	R	L	60
61	Y	D	T	E	C	D	N	A	K	G	L	K	A	F	Y	D	A	I	K	Y	80
81	G	P	N	H	L	M	V	F	G	G	V	C	P	S	V	T	S	I	I	A	100
101	E	S	L	Q	G	W	N	L	V	Q	L	S	F	A	A	T	T	P	V	L	120
121	A	D	K	K	K	Y	P	Y	F	F	R	T	V	P	S	D	N	A	V	N	140
141	P	A	I	L	K	L	L	K	H	Y	Q	W	K	R	V	G	T	L	T	Q	160
161	D	V	Q	R	F	S	E	V	R	N	D	L	T	G	V	L	Y	G	E	D	180
181	I	E	I	S	D	T	E	S	F	S	N	D	P	C	T	S	V	K	K	L	200
201	K	G	N	D	V	R	I	I	L	G	Q	F	D	Q	N	M	A	A	K	V	220
221	F	C	C	A	Y	E	E	N	M	Y	G	S	K	Y	Q	W	I	I	P	G	240

FIGURE 2B

241	W	Y	E	P	S	W	W	E	Q	V	H	T	E	A	N	S	S	R	C	L	260
261	R	K	N	L	L	A	A	M	E	G	Y	I	G	V	D	F	E	P	L	S	280
281	S	K	Q	I	K	T	I	S	G	K	T	P	Q	Q	Y	E	R	E	Y	N	300
301	N	K	R	S	G	V	G	P	S	K	F	H	G	Y	A	Y	D	G	I	W	320
321	V	I	A	K	T	L	Q	R	A	M	E	T	L	H	A	S	S	R	H	Q	340
341	R	I	Q	D	F	N	Y	T	D	H	T	L	G	R	I	I	L	N	A	M	360
361	N	E	T	N	F	F	G	V	T	G	Q	V	V	F	R	N	G	E	R	M	380
381	G	T	I	K	F	T	Q	F	Q	D	S	R	E	V	K	V	G	E	Y	N	400
401	A	V	A	D	T	L	E	I	I	N	D	T	I	R	F	Q	G	S	E	P	420
421	P	K	D	K	T	I	I	L	E	Q	L	R	K	I	S	L	P	L	Y	S	440
441	I	L	S	A	L	T	I	L	G	M	I	M	A	S	A	F	L	F	F	N	460
461	I	K	N	R	N	Q	K	L	I	K	M	S	S	P	Y	M	N	N	L	I	480

FIGURE 2C

481	I	L	G	G	M	L	S	Y	A	S	I	F	L	F	G	L	D	G	S	F	500
501	V	S	E	K	T	F	E	T	L	C	T	V	R	T	W	I	L	T	V	G	520
521	Y	T	T	A	F	G	A	M	F	A	K	T	W	R	V	H	A	I	F	K	540
541	N	V	K	M	K	K	K	I	I	K	D	Q	K	L	L	V	I	V	G	G	560
561	M	L	L	I	D	L	C	I	L	I	C	W	Q	A	V	D	P	L	R	R	580
581	T	V	E	K	Y	S	M	E	P	D	P	A	G	R	D	I	S	I	R	P	600
601	L	L	E	H	C	E	N	T	H	M	T	I	W	L	G	I	V	Y	A	Y	620
621	K	G	L	L	M	L	F	G	C	F	L	A	W	E	T	R	N	V	S	I	640
641	P	A	L	N	D	S	K	Y	I	G	M	S	V	Y	N	V	G	I	M	C	660
661	I	I	G	A	A	V	S	F	L	T	R	D	Q	P	N	V	Q	F	C	I	680
681	V	A	L	V	I	I	F	C	S	T	I	T	L	C	L	V	F	V	P	K	700
701	L	I	T	L	R	T	N	P	D	A	A	T	Q	N	R	R	F	Q	F	T	720

FIGURE 2D

721	Q	N	Q	K	K	E	D	S	K	T	S	T	S	V	T	S	V	N	Q	A	740
741	S	T	S	R	L	E	G	L	Q	S	E	N	H	R	L	R	M	K	I	T	760
761	E	L	D	K	D	L	E	E	V	T	M	Q	L	Q	D	T	P	E	K	T	780
781	T	Y	I	K	Q	N	H	Y	Q	E	L	N	D	I	L	N	L	G	N	F	800
801	T	E	S	T	D	G	G	K	A	I	L	K	N	H	L	D	Q	N	P	Q	820
821	L	Q	W	N	T	T	E	P	S	R	T	C	K	D	P	I	E	D	I	N	840
841	S	P	E	H	I	Q	R	R	L	S	L	Q	L	P	I	L	H	H	A	Y	860
861	L	P	S	I	G	G	V	D	A	S	C	V	S	P	C	V	S	P	T	A	880
881	S	P	R	H	R	H	V	P	P	S	F	R	V	M	V	S	G	L			898

FIGURE 3B

841	CCGGGATGGTACGAGCCTGCGTGGTGGGAGCAGGTGCATGTGGAGGCCAATTCTCAGC	900
901	TGCCGTGCGCAGAAAGCCTCCTGGCTGCCATGGAAGGTTACATCGGAGTGGACTTTGAGCCC	960
961	CTGAGCTCCAAACAAAATCAAGACCATCTCAGGGAAGACTCCACAGCAGTATGAAAGAGAG	1020
1021	TACAAACAGCAAACGTTTCAGGCGTGGGGCCCCAGCAAGTTCATGGGTACGCCCTACGATGGG	1080
1081	ATCTGGGTTCATCGCCAAAGACCCCTACAGAGGGCCATGAGACACTGCATGCCAGTAGCAGG	1140
1141	CACCAGCGGATCCAGGACTTCAACTACACAGACCACACGCTGGGCAAAAATCATCCTCAAT	1200
1201	GCCATGAACGAGACCAACTTCTTCGGGGTCACGGGTCAAAGTTGTGTTCCGGAAACGGGGAG	1260
1261	AGAAATGGGAACCATTAATTTACTCAATTTCAAAGACAGCAGAGAGGTGAAGTTCGGCGAA	1320
1321	TACAACGGGTGGCTGACACACTGGAGATCATCAATGACACCCATAAGGTTCCAGGGGTCC	1380
1381	GAGCCACCCAAAGGACAAAGACCATCATTTCTGGAGCAGCTTCGGAAGATCTCGCTTCCACTG	1440
1441	TATAGCATCCTGTCCGCTCTCACCATCCCTCGGCATGATCATGGCCAGCGCCTTCTCTCTTC	1500
1501	TTCAACATCAAGAACCCGGAACCAAAAGCTGATTAAGATGTCAAGCCCCCTACATGAACAAC	1560
1561	CTCATCATCCTGGGAGGAATGCTGTCTTATGCATCCATCTTCTCTCTTTTGGCCCTCGATGGG	1620
1621	TCCTTCGTCTCAGAAAAGACCTTTTGAAACACTCTGACACGGTCCGGACCTTGGATTCTCACC	1680

FIGURE 3C

1681	GTGGGCTACAACTGCCCTTTGGGGCCATGTTTGCAAAGACCTGGAGGGTCCATGCCATC	1740
1741	TTCAAAAATGTGAAGATGAAGAAGAAAGATCATCAAAGACCAGAAGCTGCTTGTAATTGTG	1800
1801	GGGGGCAATGCTGCTCATCGACCTGTGCATCCTTGATCTGTTGGCAGGCTGTGGACCCCTTG	1860
1861	CGGAGGACAGTAGAGAGGTACAGCATGGAGCCGGACCCAGCAGGCCGGGACATCTCCATC	1920
1921	CGCCCATTGCTGGAACACTGCGAAAAACACCATGACCATCTGGCTTGGCATTTGTCTAC	1980
1981	GCCTACAAGGGGCTCCTCATGTCTATTCGGTTGTTTCTTGGCATGGGAAACCCGCAATGTG	2040
2041	AGCATCCCTGCCCTCAACGACAGCAAGTACATCGGCATGAGTGTGTACAATGTGGGGATC	2100
2101	ATGTGCATCATCGGGGCTGCTGTCTCCTTCCTGACGCGTGACCAGCCCAACGTGCAGTTC	2160
2161	TGCATCGTGGCCCTGGTCAATCATCTTCTGCAGCACCATCACCTCTGCTGCTGTTGTG	2220
2221	CCAAAGCTCATTTACTCTGAGGACAAACCCCTGACGCAGCCACTCAGAACAGCGGTTCCAG	2280
2281	TTCACACAGAAACCAGAAAGAAAGATTCGAAGACCTCCACTTCAGTCAACCAGCGTGAAC	2340
2341	CAGGCGAGCACGTCA CGCCTGGAGGACTGCAGTCAGAAACCAACCGCCTTCGAATGAAG	2400
2401	ATCACAGAGCTGGACAAAGACTTGGAAGAAGTCAACCATGCAGCTACAAGACACACCAGAG	2460
2461	AAGACCACATACATAAACAGAAATCACTACCAAGAGCTCAACGACATCCTCAGCTTGGGC	2520

FIGURE 3D

2521	AAC TTCACAGAGACACAGATGGAGGAAAAGGCCATTCTAAAAAATCACCTCGATCAAAAC	2580
2581	CCCCAGCTCCAGTGGAAACACGACAGAGCCCCTCAAGAACATGCAAAAGACCCCATAGAAAGAC	2640
2641	ATCAAACTCCCCGGAGCACATCCAGCGCCGGCTGTGCTCCAGCTCCCCCATCCTTCACCAC	2700
2701	GCCTACCTCCCATCCATCGGAGGCGTGGATGCCAGCTGCGTCAGCCCCCTGTGTGTCAGCCCT	2760
2761	ACCGCCAGCCCTCGCCACAGACACGTACCACCCCTCCTTCCGAGTCATGGTCTCGGGCCCTG	2820
2821	<u>TAG</u>	2823

FIGURE 4A

1	M	A	S	P	P	S	S	G	Q	P	R	P	P	P	P	P	P	P	A	20	
21	R	L	L	L	P	L	L	L	S	L	L	L	W	L	A	P	G	A	W	G	40
41	W	T	R	G	A	P	R	P	P	P	S	S	P	P	P	L	S	I	M	G	60
61	M	P	L	T	K	E	V	A	K	G	S	I	G	R	G	V	L	P	A	V	80
81	E	L	A	I	E	Q	I	R	N	E	S	L	L	R	P	Y	F	L	D	L	100
101	R	L	Y	D	T	E	C	D	N	A	K	G	L	K	A	F	Y	D	A	I	120
121	K	Y	G	P	N	H	L	M	V	F	G	G	V	C	P	S	V	T	S	I	140
141	I	A	E	S	L	Q	G	W	N	L	V	Q	L	S	F	A	A	T	T	P	160
161	V	L	A	D	K	K	K	Y	P	Y	F	F	R	T	V	P	S	D	N	A	180
181	V	N	P	A	I	L	K	L	L	K	H	F	R	W	R	R	V	G	T	L	200
201	T	Q	D	V	Q	R	F	S	E	V	R	N	D	L	T	G	V	L	Y	G	220
221	E	D	I	E	I	S	D	T	E	S	F	S	N	D	P	C	T	S	V	K	240
241	K	L	K	G	N	D	V	R	I	I	L	G	Q	F	D	Q	N	M	A	A	260

FIGURE 4B

261	K	V	F	C	C	A	F	E	E	S	M	F	G	S	K	Y	Q	W	I	I	280
281	P	G	W	Y	E	P	A	W	E	Q	V	H	V	E	A	N	S	S	R		300
301	C	L	R	R	S	L	L	A	A	M	E	G	Y	I	G	V	D	F	E	P	320
321	L	S	S	K	Q	I	K	T	I	S	G	K	T	P	Q	Q	Y	E	R	E	340
341	Y	N	S	K	R	S	G	V	G	P	S	K	F	H	G	Y	A	Y	D	G	360
361	I	W	V	I	A	K	T	L	Q	R	A	M	E	T	L	H	A	S	S	R	380
381	H	Q	R	I	Q	D	F	N	Y	T	D	H	T	L	G	K	I	I	L	N	400
401	A	M	N	E	T	N	F	F	G	V	T	G	Q	V	V	F	R	N	G	E	420
421	R	M	G	T	I	K	F	T	Q	F	Q	D	S	R	E	V	K	V	G	E	440
441	Y	N	A	V	A	D	T	L	E	I	I	N	D	T	I	R	F	Q	G	S	460
461	E	P	P	K	D	K	T	I	I	L	E	Q	L	R	K	I	S	L	P	L	480
481	Y	S	I	L	S	A	L	T	I	L	G	M	I	M	A	S	A	F	L	F	500
501	F	N	I	K	N	R	N	Q	K	L	I	K	M	S	S	P	Y	M	N	N	520
521	L	I	I	L	G	M	L	S	Y	A	S	I	F	L	F	G	L	D	G		540

FIGURE 4C

541	S	F	V	S	E	K	T	F	E	T	L	C	T	V	R	T	W	I	L	T	560
561	V	G	Y	T	T	A	F	G	A	M	F	A	K	T	W	R	V	H	A	I	580
581	F	K	N	V	K	M	K	K	K	I	I	K	D	Q	K	L	L	V	I	V	600
601	G	G	M	L	L	I	D	L	C	I	L	I	C	W	Q	A	V	D	P	L	620
621	R	R	T	V	E	R	Y	S	M	E	P	D	P	A	G	R	D	I	S	I	640
641	R	P	L	L	E	H	C	E	N	T	H	M	T	I	W	L	G	I	V	Y	660
661	A	Y	K	G	L	L	M	L	F	G	C	F	L	A	W	E	T	R	N	V	680
681	S	I	P	A	L	N	D	S	K	Y	I	G	M	S	V	Y	N	V	G	I	700
701	M	C	I	I	G	A	A	V	S	F	L	T	R	D	Q	P	N	V	Q	F	720
721	C	I	V	A	L	V	I	I	F	C	S	T	I	T	L	C	L	V	F	V	740
741	P	K	L	I	T	L	R	T	N	P	D	A	A	T	Q	N	R	R	F	Q	760
761	F	T	Q	N	Q	K	K	E	D	S	K	T	S	T	S	V	T	S	V	N	780
781	Q	A	S	T	S	R	L	E	G	L	Q	S	E	N	H	R	L	R	M	K	800
801	I	T	E	L	D	K	D	L	E	E	V	T	M	Q	L	Q	D	T	P	E	820

FIGURE 4D

821	K	T	T	Y	I	K	Q	N	H	Y	Q	E	L	N	D	I	L	S	L	G	840
841	N	F	T	E	S	T	D	G	G	K	A	I	L	K	N	H	L	D	Q	N	860
861	P	Q	L	Q	W	N	T	T	E	P	S	R	T	C	K	D	P	I	E	D	880
881	I	N	S	P	E	H	I	Q	R	R	L	S	L	Q	L	P	I	L	H	H	900
901	A	Y	L	P	S	I	G	G	V	D	A	S	C	V	S	P	C	V	S	P	920
921	T	A	S	P	R	H	R	H	V	P	P	S	F	R	V	M	V	S	G	L	940

FIGURE 5A

1	M	P	S	A	T	G	P	L	S	I	M	G	L	M	P	20
21	L	T	K	S	I	G	R	G	V	L	P	A	V	E	L	40
41	A	I	E	S	L	L	R	P	Y	F	L	D	L	R	L	60
61	Y	D	T	K	G	L	K	A	F	Y	D	A	I	K	Y	80
81	G	P	N	G	G	V	C	P	S	V	T	S	I	I	A	100
101	E	S	L	V	Q	L	S	F	A	A	T	T	P	V	L	120
121	A	D	K	F	F	R	T	V	P	S	D	N	A	V	N	140
141	P	A	I	H	Y	Q	W	K	R	V	G	T	L	T	Q	160
161	D	V	Q	R	N	D	L	T	G	V	L	Y	G	E	D	180
181	I	E	I	F	S	N	D	P	C	T	S	V	K	K	L	200
201	K	G	N	L	I	Q	F	D	Q	N	M	A	A	K	V	220
221	F	C	C	M	Y	G	S	K	Y	Q	W	I	I	P	G	240
241	W	Y	E	Q	V	H	T	E	A	N	S	S	R	C	L	260
261	R	K	N	E	G	Y	I	G	V	D	F	E	P	L	S	280
281	S	K	Q	G	K	T	P	Q	Q	Y	E	R	E	Y	N	300
301	N	K	R	S	K	F	H	G	Y	A	Y	D	G	I	W	320

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FIGURE 5B

321	V	I	A	K	T	L	Q	R	A	M	E	T	L	H	A	S	S	R	H	Q	340
341	R	I	Q	D	F	N	Y	T	D	H	T	L	G	R	I	I	L	N	A	M	360
361	N	E	T	N	F	F	G	V	T	G	Q	V	V	F	R	N	G	E	R	M	380
381	G	T	I	K	F	T	Q	F	Q	D	S	R	E	V	K	V	G	E	Y	N	400
401	A	V	A	D	T	L	E	I	I	N	D	T	I	R	F	Q	G	S	E	P	420
421	P	K	D	K	T	I	I	L	E	Q	L	R	K	I	S	L	P	L	Y	S	440
441	I	L	S	A	L	T	I	L	G	M	I	M	A	S	A	F	L	F	F	N	460
461	I	K	N	R	N	Q	K	L	I	K	M	S	S	P	Y	M	N	N	L	I	480
481	I	L	G	G	M	L	S	Y	A	S	I	F	L	F	G	L	D	G	S	F	500

FIGURE 5C

501	V	S	E	K	T	F	E	T	L	C	T	V	R	T	W	I	L	T	V	G	520
<div style="text-align: center;">III</div>																					
521	Y	T	A	F	G	A	M	F	A	K	T	W	R	V	H	A	I	F	K	540	
<div style="text-align: center;">IV</div>																					
541	N	V	K	M	K	K	I	I	K	D	Q	K	L	L	V	I	V	G	G	560	
<div style="text-align: center;">V</div>																					
561	M	L	I	D	L	C	I	L	I	C	W	Q	A	V	D	P	L	R	R	580	
<div style="text-align: center;">VI</div>																					
581	T	V	E	K	Y	S	M	E	P	D	P	A	G	R	D	I	S	I	R	P	600
<div style="text-align: center;">VII</div>																					
601	L	L	E	H	C	E	N	T	H	M	T	I	W	L	G	I	V	Y	A	Y	620
<div style="text-align: center;">VIII</div>																					
621	K	G	L	L	M	L	F	G	C	F	L	A	W	E	T	R	N	V	S	I	640

FIGURE 5D

641	P	A	L	N	D	S	K	Y	I	G	M	S	V	Y	N	V	G	I	M	C	660
661	I	I	G	A	A	V	S	F	L	T	R	D	Q	P	N	V	Q	F	C	I	680
681	V	A	L	V	I	I	F	C	S	T	I	T	L	C	L	V	F	V	P	K	700
701	L	I	T	L	R	T	N	P	D	A	A	T	Q	N	R	R	F	Q	F	T	720
721	Q	N	T	Q	K	L	E	D	S	K	T	S	E	M	L	L	T	C	Q	V	740
741	S	T	L	S	R	K	E	G	L	Q	V	S	E	M	L	L	T	C	Q	V	760
761	E	L	D	I	K	K	E	H	E	Y	K	P	R	D	P	P	S	V	A	T	780
781	T	L	Y	I	K	K	E	H	E	Y	K	P	R	D	P	P	S	V	A	T	800
801	T	L	Y	I	K	K	E	H	E	Y	K	P	R	D	P	P	S	V	A	T	820
821	L	S	Q	P	E	S	W	E	Q	P	E	S	W	E	Q	P	E	S	W	E	840
841	S	L	P	P	S	I	G	R	H	I	G	R	H	I	G	R	H	I	G	R	860
861	S	L	P	P	S	I	G	R	H	I	G	R	H	I	G	R	H	I	G	R	880
881	S	L	P	P	S	I	G	R	H	I	G	R	H	I	G	R	H	I	G	R	898

Figure 6A

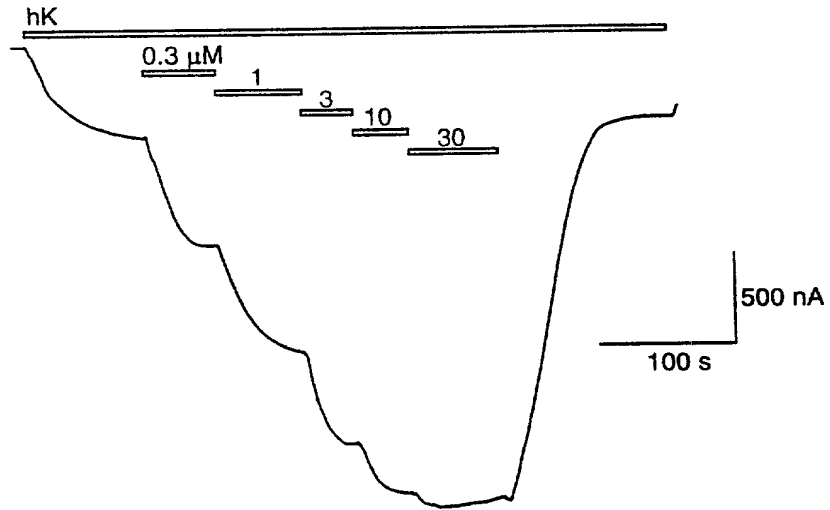


Figure 6B

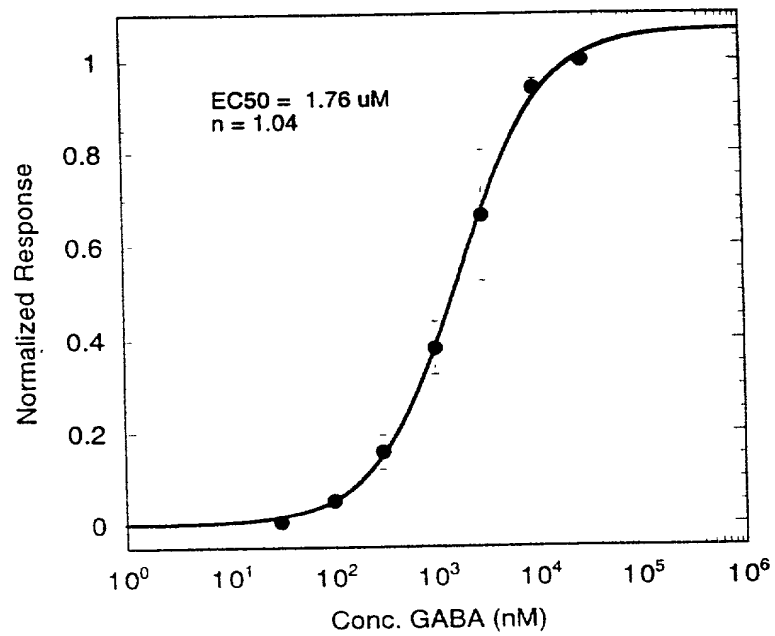


Figure 7

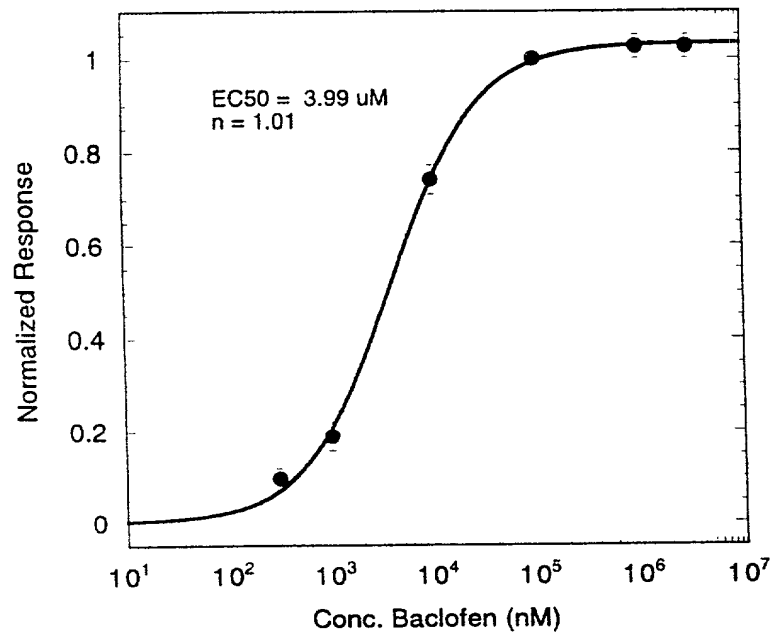


Figure 8

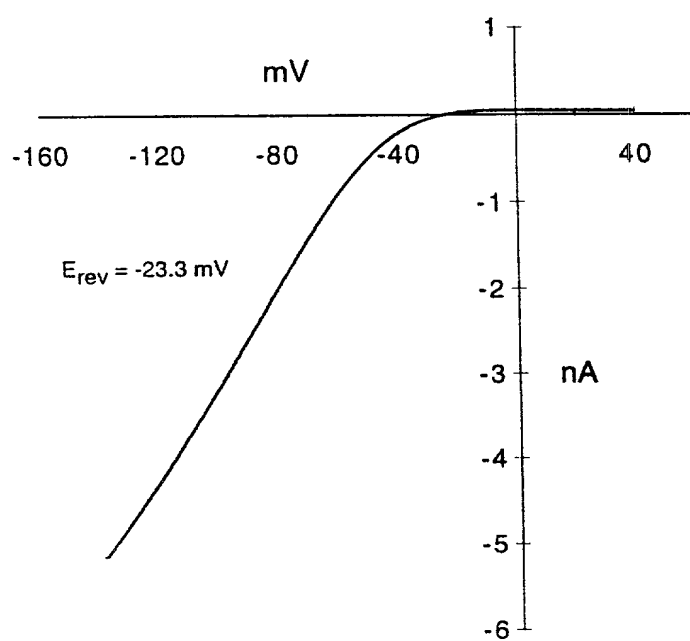


Figure 9A

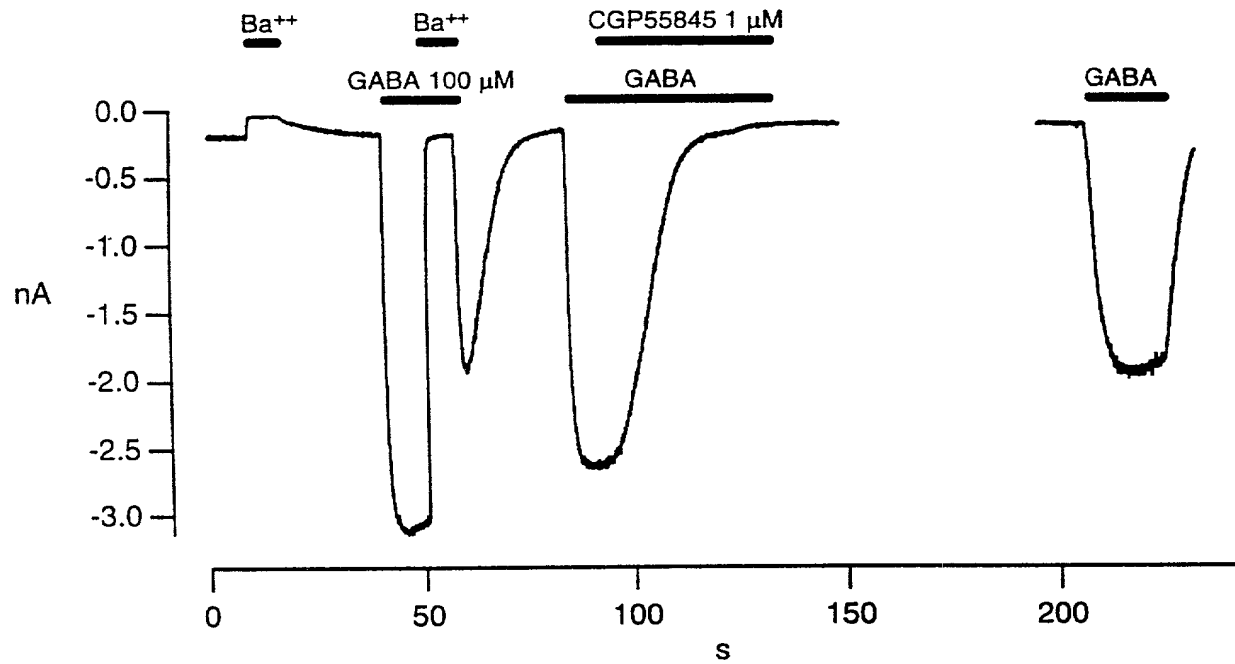


Figure 9B

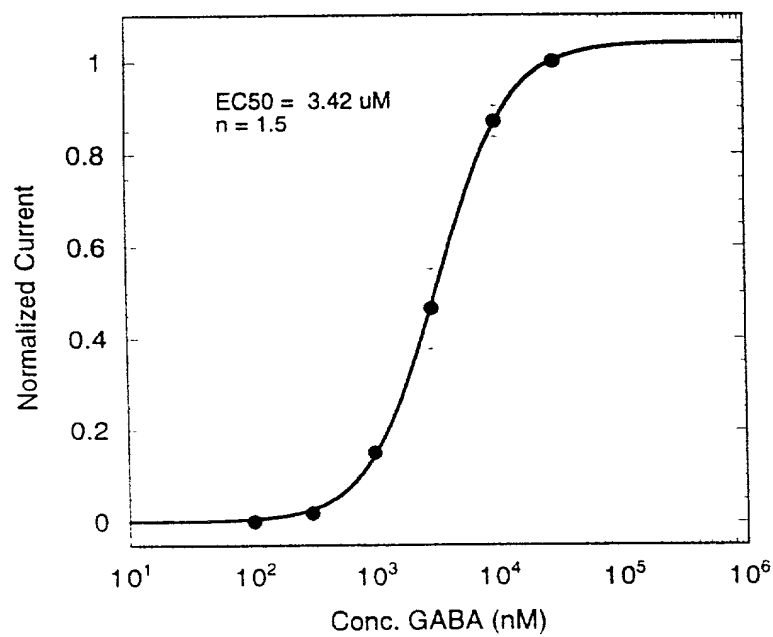


Figure10

rGABA _b R2	MASPPSSGQPRPPPPPPARLLPLLLSLLMLAPGAWGTRGAPRPPSPSP...LSIMGLMPLTK	65
rGABA _b R1bMGPGGCTPVGWPLPLLLVMAAGVAPVWASHPHLPRPHERVPPHPSSERRAVYIGALFP	60
rGABA _b R2	EVAKSTGRGVLPAVELAIEQIRN.ESLLRPYFLDLRLDYTECDNAKGLKAFYDAIKYGNHLMVFGGVC	134
rGABA _b R1b	MSGWPGGQACQPAVEMALEDVNSRRDILPDYELKLIHDSKCDPGQATKYLYELLYNDPIKIILMPG.C	129
rGABA _b R2	PSVTSIIAESLQGNLVQLSFAATTPVLADKKKYPYFFRTVPSDNAVNPAILKLLKHFRWRRVGTLTQDV	204
rGABA _b R1b	SSVSTLVAEAAARMNLIIVLSYGSSSPALSNRQRFPTFFRTHPSATLHNPRVKLFEKMGWKKIATIQOTT	199
rGABA _b R2	QRFSEVRNDLTGVLYGEDIETSDTESFNDPCTSVYKLLKGNDRRIILGQFDQNMMAKVFCFAFEESMFGS	274
rGABA _b R1b	EVFTSLDDLEERVKEAGIEITFRQFFSDPAVPVKNLKRQDARIIVGLFYETEARKVFCEVYKERLFGK	269
rGABA _b R2	KYQWIIIPGWYEPAAWWEQHVHEANSSRCLRRSLLAMEGYIGVDFEPLSSKQIKTISGKTPQQYEREYNSK	344
rGABA _b R1b	KYVWFLLIGWYADNWFKTYDPSIN...CTVEEMTEAVEGHITTEIVMLNPANTRSI SNMTSQEFV.EKLT	335
rGABA _b R2	RSVGPSKFGHY.....AYDGIWVIAKTLQRAMETLHASSRHQRIQDFNYTDHTLGKIIILNAMNETNFFG	409
rGABA _b R1b	RLKRHPETGGFQEAPLAYDAI WALALALNKTSGGGRSG..VRLEDFNYNQITTDQIYRAMNSSSFE	403
rGABA _b R2	VTGQVVF.RNGERMGTIKFTQFQDSREVKVGEYNAVADTLEIINDTIRFQGESEPPKDKTIILEQLRKISL	478
rGABA _b R1b	VSGHVVFDAAGSRMAWTLIEQLQGGSYKKIGYDSTKDDLS.WSKTDKMWGGSPADQTLVIKTFRLSQ	472
rGABA _b R2	PLYSILSALTILGMIMASAFLEFNKRNQKLIKMSPPYMNLIILGGLMSYASIFLFGLDGSEFVSEKTF	548
rGABA _b R1b	KLFISVSVLSSLGIVLAVVCLSFNIYNHVRVYIQNSQPNLNLTA VGCSLALAAVFPPLGLDGYHIGRSQF	542
rGABA _b R2	ETLCTVTRTWILTVGYTTAFGAMFAKTWRVHAI FKNVKKMK...KIIKDQKLLVIVGGMLLIDLCLICWQ	615
rGABA _b R1b	PFVCQARLWLLGLGFSLGYGSMFTKIWWVHTVFTKKEKKEWKRTLEPWKLYATVGLLVGMDVLTIAIWQ	612
rGABA _b R2	AVDPLRRTVERYSMEDPDAGRDISIRPLLEHCENTHMTIWL GIVYAYKGLLMLFGCFLAWETRNVSIPAL	685
rGABA _b R1b	IVDPLHRTIETFAKEPKEDIDVSILPQLEHCSSKKMNTWLGIFYGYKGLLLLLGIFLAYETKSVSTEKI	682
rGABA _b R2	NDSKYIGMSVYNVGIMCIIIGAAVSFLTRDQPNVQFCIVALVIIFCSTITLCLVFPVKLITLRTNPDAA	755
rGABA _b R1b	NDHRAVGMAIYNVAVLCLITAPVTMILSSQQDAAFASIAIVFSSYITLVVLFVPKMRRLITRGE....	748
rGABA _b R2	NRRFQFTQNKKEDSKTSTSVTSVNQASTSRLEGLQSENHRLRMKITELDKLEEVTMQLQDTPKTTYI	825
rGABA _b R1bWQSETQDMMKTGSS..TNNNEEKSRLL.EKENRELEKIIAEKEERVSELRHQLQSRQQLRSRR	809
rGABA _b R2	KQNHVQELNDILSLGNFTESDGGKAILKNHLDONPQLQWNTTEPSRTCKDPDIEDINSPEHIQRRLSLQL	895
rGABA _b R1b	HPPTPPDPSGGLPRGPSEPPDRLSCDGSRVHLLYK*.....	845
rGABA _b R2	PILHHAYLPSIGGVDA SCVSPCVSPTASPRHRHVPPSFRVMVSGL*.....	940

Figure 11A



Figure 11B



Figure 11C



Figure 11D



0924755-1438
065727-5577260

Figure 12A

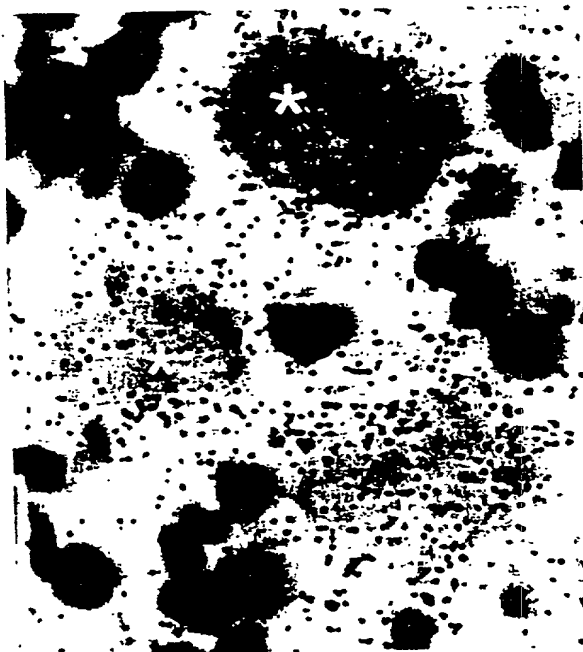


Figure 12B



0924755, 424598
965727, 9977260

Figure 13A

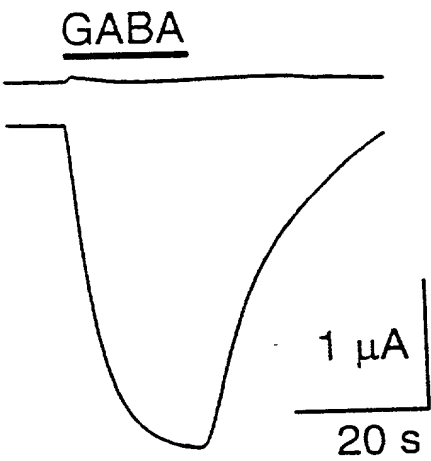


Figure 13B

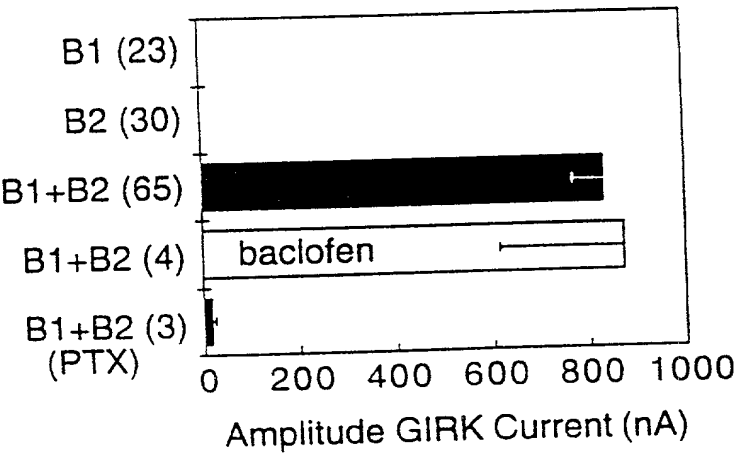


Figure 14A

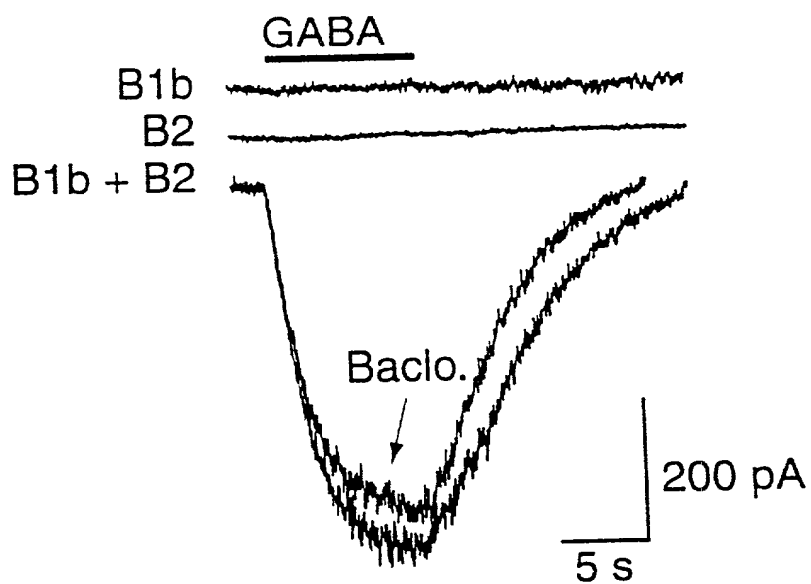


Figure 14B

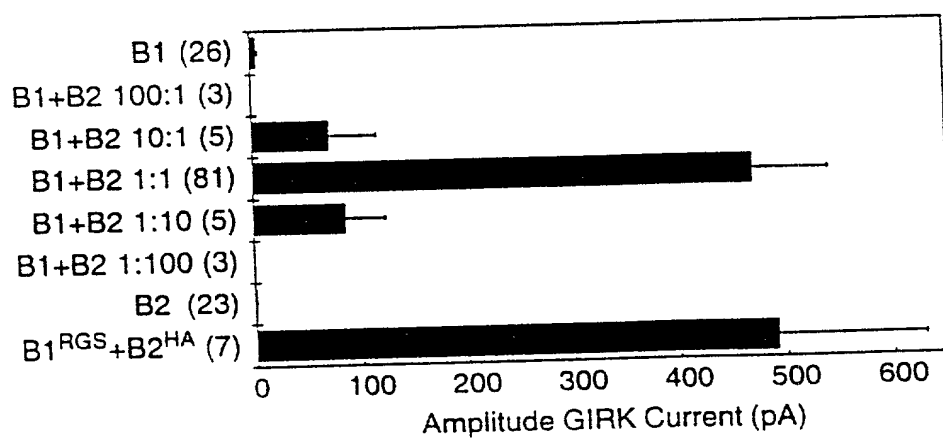


Figure 15A

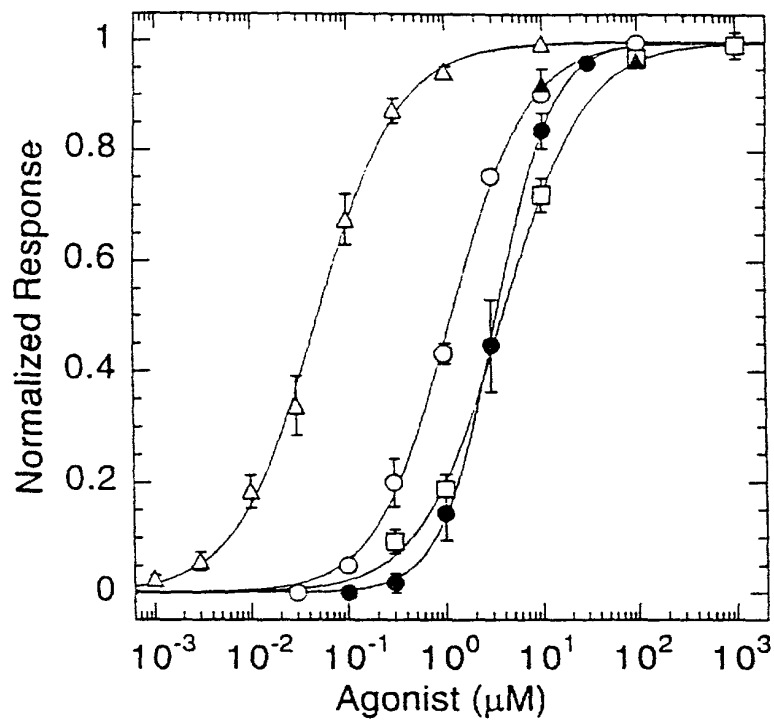


Figure 15B

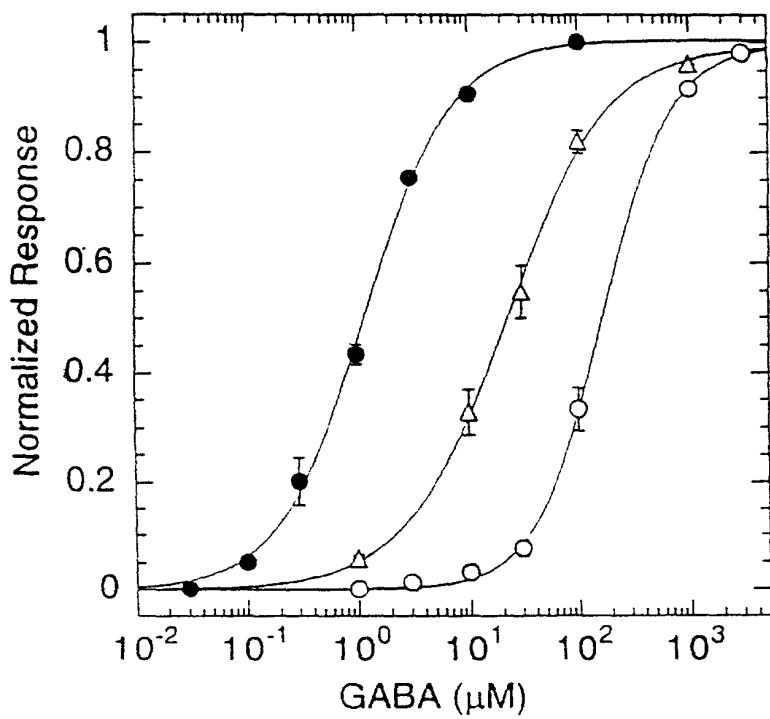


Figure 16

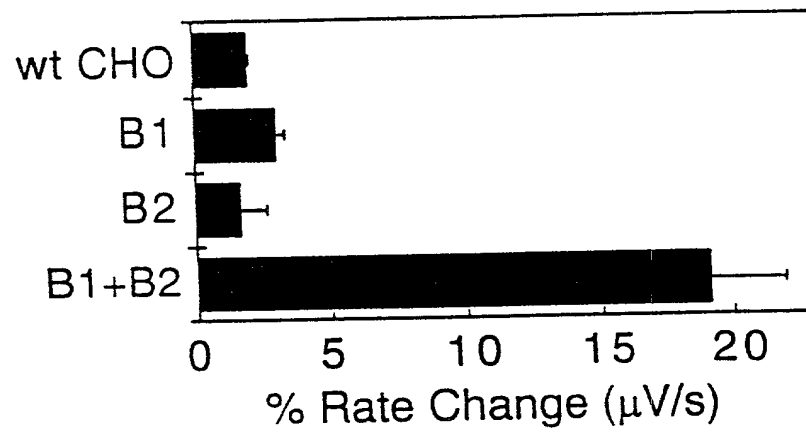


Figure 17A

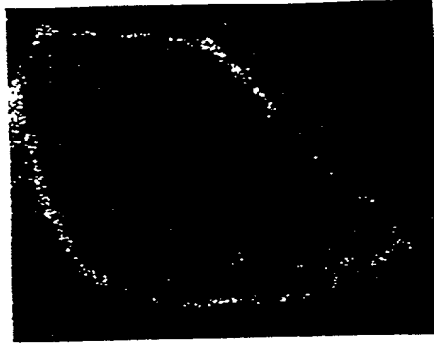


Figure 17B



Figure 17C



Figure 17D



Figure 18A

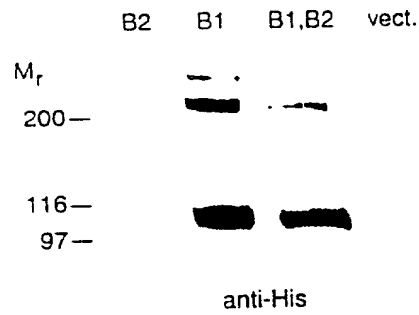


Figure 18B

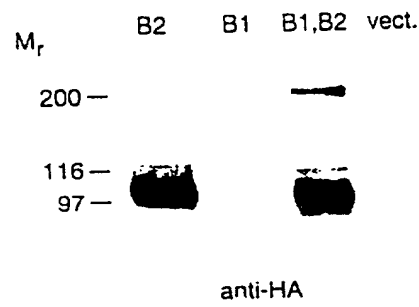
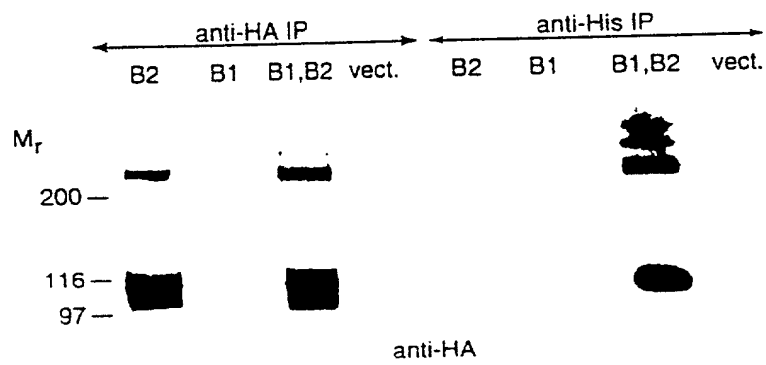
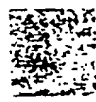


Figure 18C



Silver grain density:



+1



+2



+3



+4

FIGURE 19A



FIGURE 19B

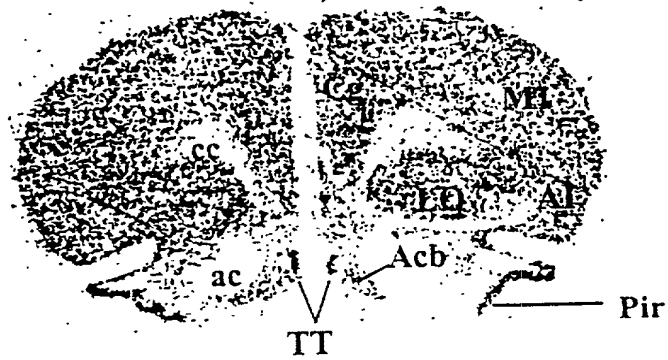
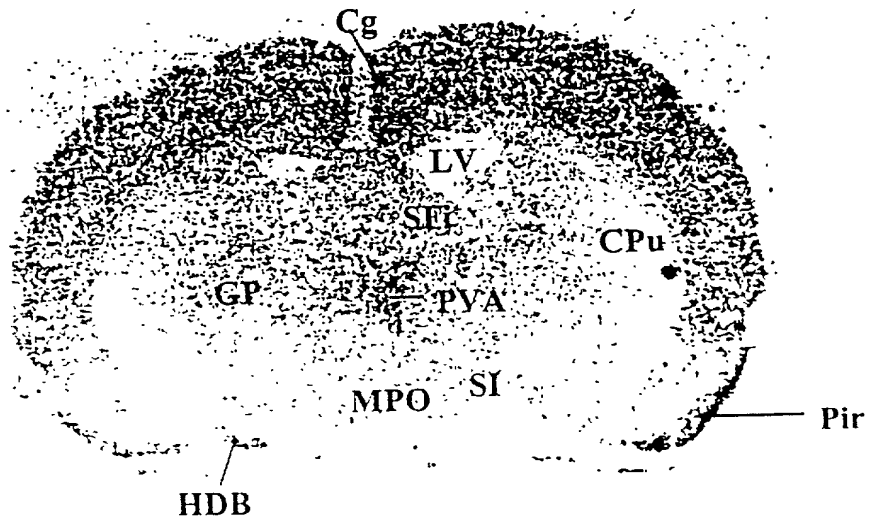


FIGURE 19C



00572T 55Z FEB 60

FIGURE 19D

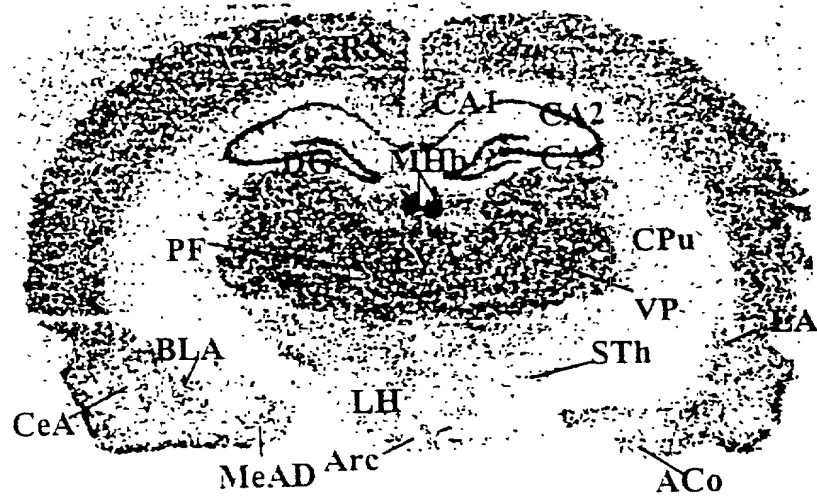


FIGURE 19E

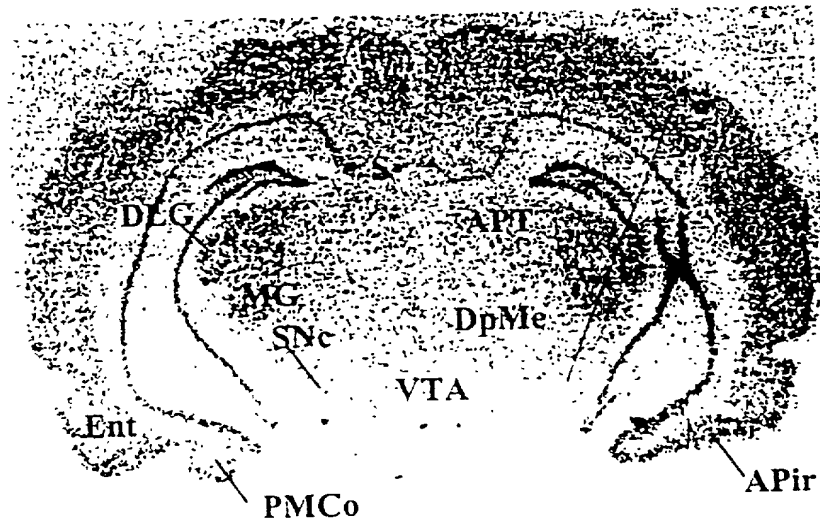
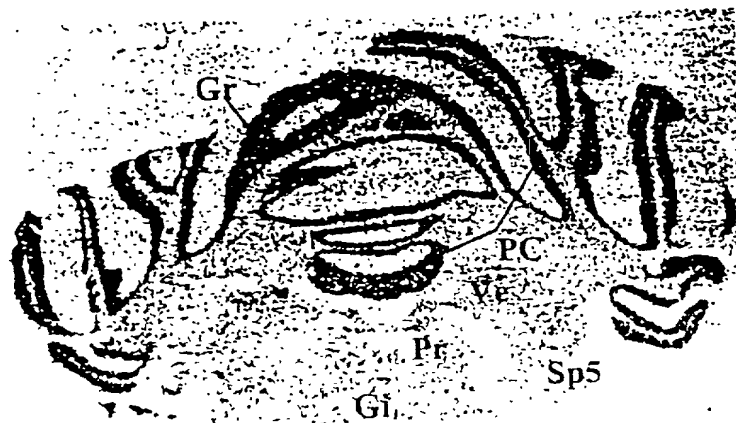


FIGURE 19F



0924755-121598

FIGURE 19G

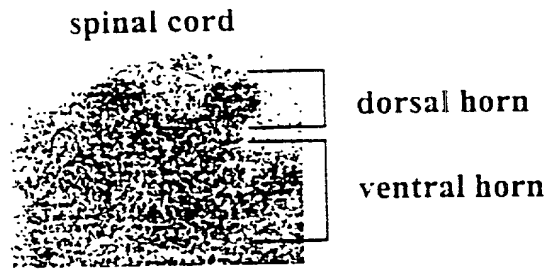


FIGURE 19H

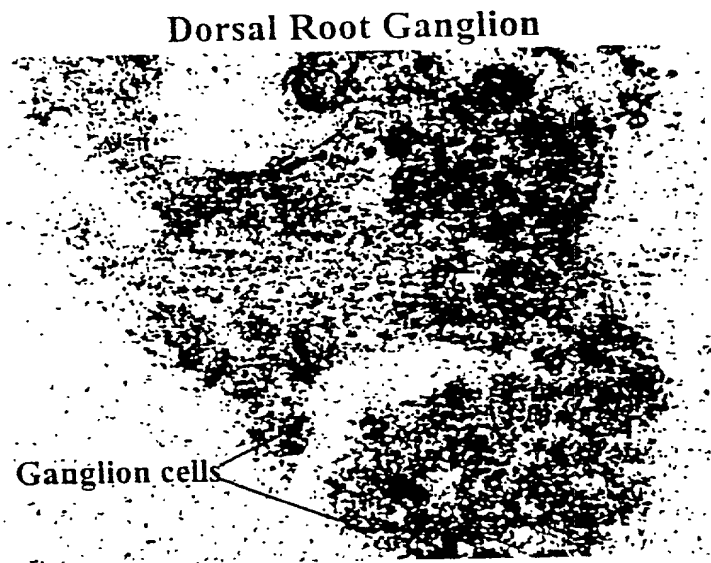
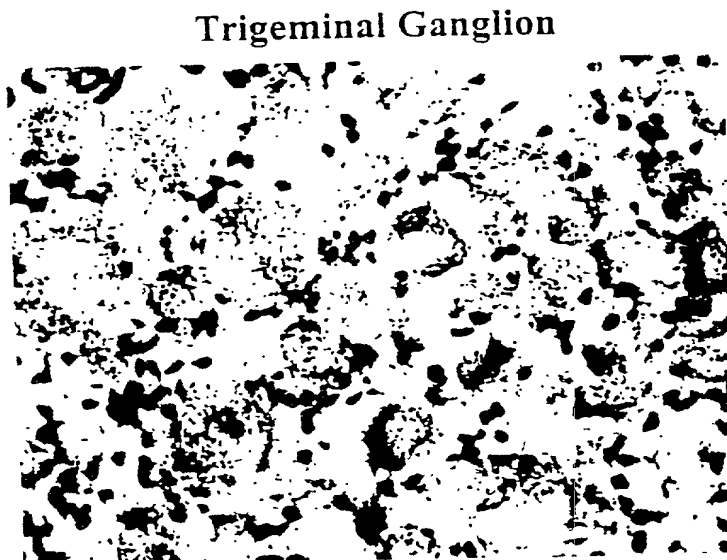


FIGURE 19I



0921155-121598

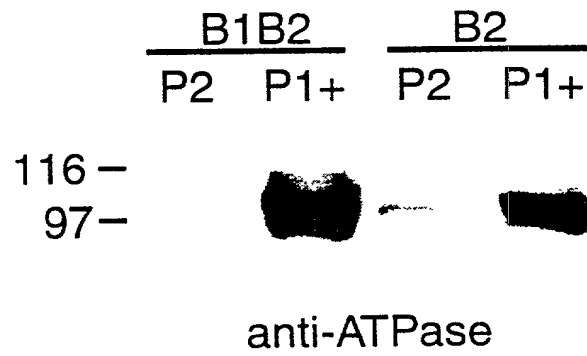
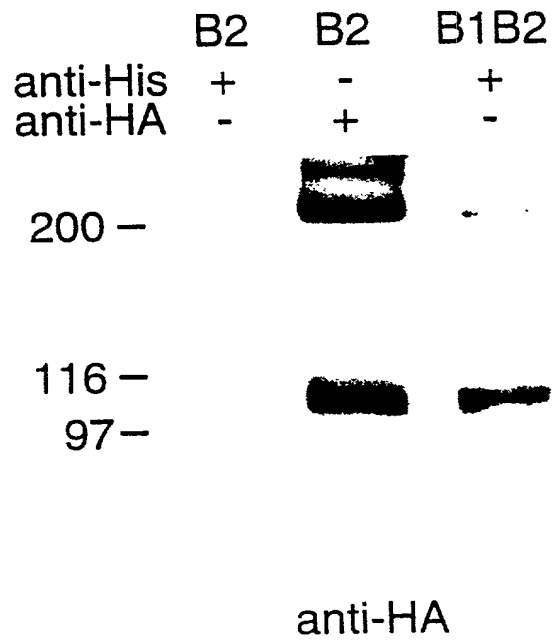
Figure 20A**Figure 20B**

Figure 20C

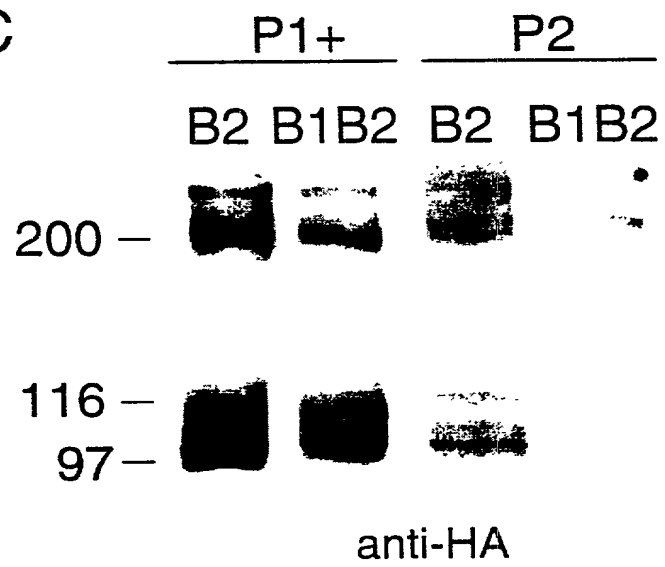


Figure 21A



Figure 21B



Figure 21C



Figure 21D



09214755, 13158
065727, 9527260

Figure 21E

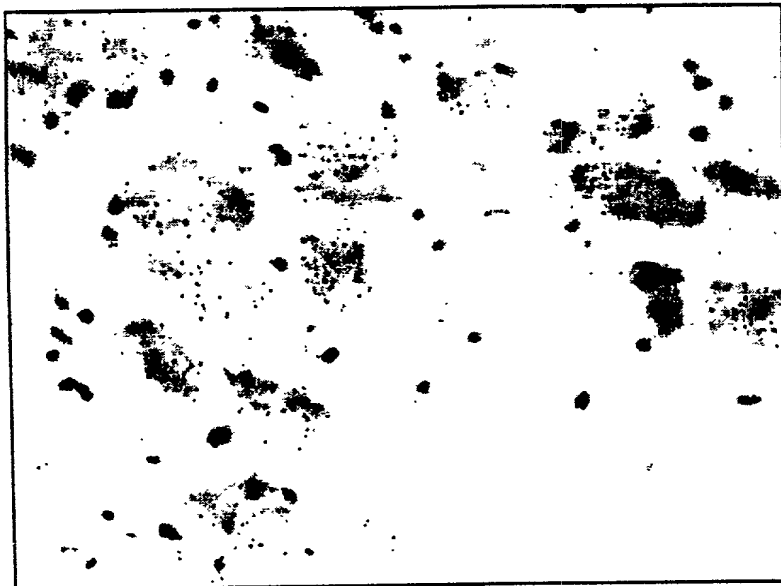
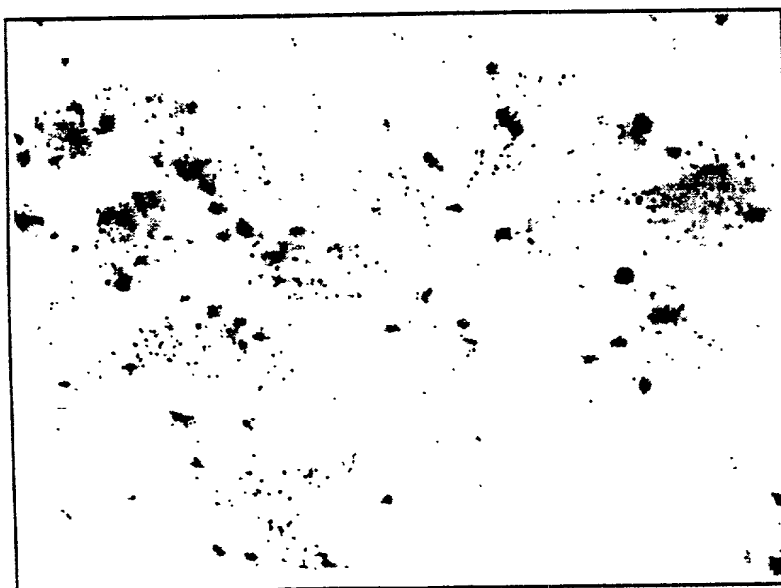


Figure 21F



09241755-124598
865421 557F260

FIGURE 22A

1 ATGGCTTCCCCGGGAGCTCCGGGCAGCCCGGGCCGCCGCCGCCGCCGCCGCCGCCGCCGCCGCC 60

61 GCGCGCCCTGCTACTGCTACTGCTGCTGCCGCTGCTGCTGCCCTCTGGCGCCCGGGCCCTGG 120

121 GGC TGGGCGGGGCGCCCCCGGCCGCCGCCAGCAGCCCGCCGCTCTCCATCATGGGC 180

181 CTCATGCCGCTCACCAAGGAGGTGGCCAAGGGCAGCATCGGGCGGGTGTGCTCCCCCGCC 240

241 GTGGAAC TGGCCATCGAGCAGATCCGCCAACGAGTCACTCCTGCGCCCCCTACTTCCTCGAC 300

301 CTGCGGCTCTATGACACGGAGTGCACAAACGCAAAAGGGTTGAAAGCCTTCTACGATGCG 360

361 ATAAAAATACGGGCGCAACCACTTGATGGTGT TGGAGGCGTCTGTCCATCCGTCACATCC 420

421 ATCAT TGCAGAGTCCCC TCCAAGCTGGAATCTGGTGCAGCTTCTTTTGTCTGCAACCACG 480

481 CCTGT TCTAGCCGATAAGAAAAAATACCCTTATTTCTTTTCGGACCGTCCCATCAGACAAAT 540

541 GCGGTGAATCCAGCCATTC TGAAGTTGCTCAAGCACTACCAGTGGAAAGCGCGTGGGCACG 600

601 CTGACGCAAGACGTTCA GAGGTTCTCTGAGGTGCGGAATGACCTGACTGGAGTTCTGTAT 660

661 GCGGAGGACATTGAGATTT CAGACACCGAGAGCTTCTCCAACGATCCC TGTACCAGTGTC 720

721 AAAAAGCTGAAGGGAATGATGTGCGGATCATCCTTGGCCAGTTTGACCAGAAATATGGCA 780

781 GCAAAAAGTGTCTGTTGTGCATACGAGGAGAACATGTATGGTAGTAAATATCAGTGGATC 840

FIGURE 22B

841	ATTCCGGGCTGGTACGAGCCTTCTTGGTGGGAGCAGGTGCACACGGAAGCCAACTCATCC	900
901	CGCTGCCCTCCGGAAGAAATCTGCTTGCTGCCATGGAGGGCTACATTGGCGTGGATTTCGAG	960
961	CCCCTGAGCTCCAAGCAGATCAAGACCATCTCAGGAAAGACTCCACAGCAGTATGAGAGA	1020
1021	GAGTACAACAAGCGGTCAAGCGTGGGGCCCCAGCAAGTTCCACGGGTACGCCCTACGAT	1080
1081	GGCATCTGGGTCA TCGCCAAGACACTGCAGAGGGCCATGGAGACACTGCATGCCAGCAGC	1140
1141	CGGCACCAGCGGATCCAGGACTTCAACTACACGGACCACACCGCTGGGCAGGATCATCCTC	1200
1201	AATGCCATGAACGAGACCAACTTCTTCGGGGTCA CGGGTCAAGTTGTATTCCGGGAATGGG	1260
1261	GAGAGAA TGGGACCATTAAATT TACTCAATT TCAAGACAGCAGGAGGTGAAGGTGGGA	1320
1321	GAGTACAACGCTGTGGCCGACACACTGGAGATCATCAATGACACCATCAGGTTCCAAAGGA	1380
1381	TCCGAACCAACCAAAGACAAGACCATCATCCTGGAGCAGCTGCCGGAAGATCTCCCTACCT	1440
1441	CTCTACAGCATCCTCTCTGCCCCTCACCATCCTCGGGATGATCATG GCCAGTGCTTTTCTC	1500
1501	TTCTTCAACATCAAGAACCGGAATCAGAAGCTCATAAAGATGTCGAGTCCATACATGAAC	1560
1561	AACCTTATCATCCTTGGAGGGATGCTTTCCTATGCTTCCATAATTTCTCTTTTGGCCTTGAT	1620
1621	GGATCCTTTGTCTCTGAAAAGACCCTTTGAACACTTTGCACCGTCAAGGACCTGGATTCTC	1680

FIGURE 22C

1681	ACCGTGGGCTACAGACCGCTTTTGGGGCCATGTTTGCAAAGACCTGGAGAGTCCACGCC	1740
1741	ATCTTCAAAAATGTGAAAAATGAAAGAAGAGATCATCAAGGACCAGAAACTGCTTGTGATC	1800
1801	GTGGGGGGCATGCTGCTGATCGACCTGTGTATCCTGATCTGCTGGCAGGCTGTGGACCCC	1860
1861	CTGCCAAGGACAGTGGAGAAAGTACAGCATGGAGCCGACCCAGCAGGACGGGATATCTCC	1920
1921	ATCCGCCCTCTCCTGGAGCACTGTGAGAACACCCATATGACCATCTGGCTTGGCATCGTC	1980
1981	TATGCCCTACAAGGACTTCTCATGTTGTTCCGGTTGTTTCTTAGCTTGGGAGACCCGCAAC	2040
2041	GTCAGCATCCCCGCACTCAACGACAGCAAGTACATCGGGATGAGTGTCTACAACGTGGGG	2100
2101	ATCATGTGCATCATCGGGGCCGCTGTCTCCTTGACCCGGGACCAGCCCAATGTGCAG	2160
2161	TTCCTGCATCGTGGCTCTGGTCAATCATCTTCTGCAGCACCATCACCCCTCTGCCCTGGTATTC	2220
2221	GTGCCGAAGCTCATCACCCCTGAGAAACAAACCCAGATGCAGCAACGCAGAACAGCGGATTC	2280
2281	CAGTTCACTCAGAAATCAGAAAGAAAGATTCTAAAACGTCCACCTCGGTCAACCAGTGTG	2340
2341	AACCAAGCCAGCACATCCCGCTGGAGGGCCCTACAGTCAGAAAACCATCGCCCTGCCGAATG	2400
2401	AAGATCACAGAGCTGGATAAAGACTTGGGAAGAGGTCACCATGCAGCTGCAGGACACACCA	2460
2461	GAAAAGACCCTACATTAAACAGAACCCACTACCAAGAGCTCAATGACATCCTCAACCTG	2520

FIGURE 22D

2521	GGAAACTTCACTGAGAGCACAGATGGAGGAAAGGCCATTTTAAAAAATCACCTCGATCAA	2580
2581	AATCCCCAGCTACAGTGGAAACACACAGAGCCCCTCTCGAACATGCAAAGATCCTATAGAA	2640
2641	GATATAAACTCTCCAGAAACACATCCAGCGTCGGCTGTCCCTCCAGCTCCCCCATCCTCCAC	2700
2701	CACGCCCTACCTCCCATCCATCGGAGGCGTGGACGCCAGCTGTGTTCAGCCCCCTGCCGTCAGC	2760
2761	CCCACCGCCAGCCCCCGCCACAGACATGTGCCACCCCTCCTTCCGAGTCATGGTCTCGGGC	2820
2821	CTGTAA	2826

FIGURE 23A

1	M	A	S	P	R	S	S	G	Q	P	G	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P	P
21	A	R	L	L	L	L	L	L	L	P	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L	L
41	G	W	A	R	G	A	P	R	P	P	P	S	S	S	P	L	S	I	M	G							
61	L	M	P	L	T	K	E	V	A	K	G	S	I	G	R	G	V	L	P	A							
81	V	E	L	A	I	E	Q	I	R	N	E	S	L	L	R	P	Y	F	L	D							
101	L	R	L	Y	D	T	E	C	D	N	A	K	G	L	K	A	F	Y	D	A							
121	I	K	Y	G	P	N	H	L	M	V	F	G	G	V	C	P	S	V	T	S							
141	I	I	A	E	S	L	Q	G	W	N	L	V	Q	L	S	F	A	A	T	T							
161	P	V	L	A	D	K	K	K	Y	P	Y	F	F	R	T	V	P	S	D	N							
181	A	V	N	P	A	I	L	K	L	L	K	H	Y	Q	W	K	R	V	G	T							
201	L	T	Q	D	V	Q	R	F	S	E	V	R	N	D	L	T	G	V	L	Y							
221	G	E	D	I	E	I	S	D	T	E	S	F	S	N	D	P	C	T	S	V							
241	K	K	L	K	G	N	D	V	R	I	I	L	G	Q	F	D	Q	N	M	A							
261	A	K	V	F	C	C	A	Y	E	E	N	M	Y	G	S	K	Y	Q	W	I							

FIGURE 23B

	49/51																				
281	I	P	G	W	Y	E	P	S	W	E	Q	V	H	T	E	A	N	S	S	300	
301	R	C	L	R	K	N	L	L	A	A	M	E	G	Y	I	G	V	D	F	E	320
321	P	L	S	S	K	Q	I	K	T	I	S	G	K	T	P	Q	Q	Y	E	R	340
341	E	Y	N	N	K	R	S	G	V	G	P	S	K	F	H	G	Y	A	Y	D	360
361	G	I	W	V	I	A	K	T	L	Q	R	A	M	E	T	L	H	A	S	S	380
381	R	H	Q	R	I	Q	D	F	N	Y	T	D	H	T	L	G	R	I	I	L	400
401	N	A	M	N	E	T	N	F	F	G	V	T	G	Q	V	F	R	N	G	420	
421	E	R	M	G	T	I	K	F	T	Q	F	Q	D	S	R	E	V	K	V	G	440
441	E	Y	N	A	V	A	D	T	L	E	I	I	N	D	T	I	R	F	Q	G	460
461	S	E	P	P	K	D	K	T	I	I	L	E	Q	L	R	K	I	S	L	P	480
481	L	Y	S	I	L	S	A	L	T	I	L	G	M	I	M	A	S	A	F	L	500
501	F	F	N	I	K	N	R	N	Q	K	L	I	K	M	S	S	P	Y	M	N	520
521	N	L	I	I	L	G	G	M	L	S	Y	A	S	I	F	L	F	G	L	D	540
541	G	S	F	V	S	E	K	T	F	E	T	L	C	T	V	R	T	W	I	L	560

FIGURE 23C

561 T V G Y T T A F G A M F A K T W R V H A 580
 581 I F K N V K M K K K I I K D Q K L L V I 600
 601 V G G M L L I D L C I I L I C W Q A V D P 620
 621 L R R T V E K Y S M E P D P A G R D I S 640
 641 I R P L L E H C E N T H M T I W L G I V 660
 661 Y A Y K G L L M L F G C F L A W E T R N 680
 681 V S I P A L N D S K Y I G M S V Y N V G 700
 701 I M C I I G A A V S F L T R D Q P N V Q 720
 721 F C I V A L V I I F C S T I T L C L V F 740
 741 V P K L I T L R T N P D A A T Q N R R F 760
 761 Q F T Q N Q K K E D S K T S T S V T S V 780
 781 N Q A S T S R L E G L Q S E N H R L R M 800
 801 K I T E L D K D L E E V T M Q L Q D T P 820
 821 E K T T Y I K Q N H Y Q E L N D I L N L 840

FIGURE 23D

841	G	N	F	T	E	S	T	D	G	G	K	A	I	L	K	N	H	L	D	Q	860
861	N	P	Q	L	Q	W	N	T	T	E	P	S	R	T	C	K	D	P	I	E	880
881	D	I	N	S	P	E	H	I	Q	R	R	L	S	L	Q	L	P	I	L	H	900
901	H	A	Y	L	P	S	I	G	G	V	D	A	S	C	V	S	P	C	V	S	920
921	P	T	A	S	P	R	H	R	H	V	P	P	S	F	R	V	M	V	S	G	940
941	L																			941	